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The identification of effectors of retinal cell fate determination through single cell transcriptomics

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DEDICATION

This dissertation is dedicated to my grandpa, the first Doc Goetz. Grandpa Doc is the total package: not only is he resilient and tough, but also quick with a joke or an offering of his own eye if it could help my research. The past few years have been so hard for our entire family, Grandpa Doc especially, and I regret not being there for him more. Hopefully this offering makes up for a bit of it.

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CHAPTER 1. GENERAL INTRODUCTION

1. Background information

1.1 Introduction to the retina

The proper integration of sensory information as vital as vision requires a precisely functioning instrument. In vertebrates, this process begins in the retina. This photosensitive tissue at the back of the eye is responsible for phototransduction – the conversion of light energy to neural signals – and the initial processing of visual information before the signals are passed on to the brain (Burns and Arshavsky, 2005). Specialized retinal neurons contribute to basic signal modulation even before those features are forwarded on to the visual centers of the brain. These nerve cells range from the light-sensing photoreceptors with their unique outer segments to the retinal ganglion cells whose long axons must connect with specific partners in the brain. Interestingly, each mature retinal neuron emanates from the same retinal progenitor population and precisely integrates into the retinal circuitry to perform its own specific role in vision.

Understanding the factors directing each progenitor cell to its final fate as a specialized neuron is important for multiple reasons. First, the factors that drive the transition from an uncommitted progenitor cell to a fully functioning neuron may be shared among developing central nervous system (CNS) neurons beyond the retina. Knowledge regarding how these factors function in concert would, therefore, inform our understanding of how different neuronal populations are generated. Second, a greater understanding of how each retinal progenitor cell determines its fate is of great interest not only for developmental biologists, but also for those interested in diseases of the visual system. Many degenerative conditions affecting the retina could potentially be treated through cellular replacement therapy. These cell-based treatments involve either regenerating lost retinal neurons in the diseased tissue or injecting new cells that have been produced in culture to replace those that have been lost (Schmeer et al., 2012). Therapies such as these require a thorough knowledge of how to generate a significant number of different types of neurons to replace those that are deteriorating. We can only uncover all of the factors to be used in these cutting edge replacement strategies through studies of the mechanisms behind retinal cell fate decisions.

1.2 Cell types that comprise the retina

1.2.1 Photoreceptors

Although much remains to be elucidated regarding how the precise retinal circuitry is established, there is a significant amount that is understood about the

different cells that make up the retina. The retina possesses a laminar organization that is well conserved throughout vertebrate evolution. It is composed of six major types of neurons and one type of glia, all of which are located within specific layers of the retina (Masland, 2012; Rodieck, 1998). The two types of photoreceptors, rods and cones, array themselves at the outer portion of the eye in the outer nuclear layer (ONL) (see Figure 1A). As their name suggests, photoreceptors function as the primary sensory neurons in the retina, converting light photons into chemical signals that can then be passed on to interneurons in the inner nuclear layer (INL) for further processing. In general, photoreceptors are depolarized in the absence of light, which leads to a constant sodium influx and release of the neurotransmitter glutamate to activate downstream interneurons (Yau, 1994). Conversely, when stimulated by light, photoreceptors hyperpolarize and cease to release glutamate (Yau, 1994). Rod photoreceptors, containing the photopigment rhodopsin, are best suited for low light conditions and distinguish between light and dark (Morrow et al., 1998).

Cone photoreceptor cells are distinguishable from rod photoreceptors by the presence of specialized opsins that react to ranges of brighter light at different wavelengths (Bruhn and Cepko, 1996; Masland, 2012). Although different numbers of opsins are present in various vertebrates, many, including humans, possess three distinct opsins – L or red opsins, which have a peak spectral sensitivity of 560nm; M or green opsins, with a peak sensitivity of 530nm; and S or blue opsins, with a peak sensitivity around 430nm (Cheung et al., 2013). Possibly owing to the fact that mice are nocturnal animals that heavily rely on their sense of smell, murine retinas

contain only the second two varieties of opsins, although multiple cone phenotypes can arise from the expression of different combinations of opsins (Applebury et al., 2000). The diurnal chick, while possessing the same three cone opsins noted in primates, contains an additional violet opsin, as well as a specialized double cone that generally expresses red opsin in each of its two connected cell bodies (Bruhn and Cepko, 1996; Enright et al., 2015). Observations of the generation of specific cone subtypes in vertebrate species have indicated that subtype-specific transcription factors lead to the early generation of the more numerous longer-wavelength opsins considerably ahead of their more rare short-wavelength counterparts during retinal development (Bruhn and Cepko, 1996; Cheung et al., 2013; Enright et al., 2015).

1.2.2. Interneurons

Regardless of each photoreceptor's range of excitability, once a given photoreceptor hyperpolarizes in response to photon absorption, their glutamate inhibition decreases, allowing for signals to pass from the ONL through to the interneurons of the INL (Masland, 2001a; Masland and Raviola, 2000). As opposed to sensory or output neurons, interneurons are recognized by their role as intermediate relays for circuits between those two types. While the specific responsibilities of interneurons vary based on their connectivity, retinal interneurons are specifically responsible for mediating the communication between the sensory neurons (photoreceptors) and output neurons (retinal ganglion cells) of the tissue (Masland, 2001a, 2001b). There are three different interneurons in the

retina: horizontal cells arrayed in a mosaic pattern along the apical side of the INL neighboring the ONL, bipolar interneurons stretching from the apical to basal edges of the INL, and the diverse amacrine interneurons present in both the INL and displaced throughout the ganglion cell layer (GCL) (Masland, 2001a).

Among the interneuron cell classes, horizontal cells function to even out signals from bright and dim areas of the visual field using a phenomenon known as lateral inhibition (Masland, 2012; Thoreson and Mangel, 2012). The lateral inhibition of cones by horizontal cells leads photoreceptors to react to their receptive fields with a center-surround organization that, while activated by light, is also inhibited by the activation of neighboring photoreceptors. The phenomenon of lateral inhibition leads to better acuity and edge detection even in the presence of bright stimuli (Thoreson and Mangel, 2012). In general, mammals possess two types of horizontal cells, distinguishable by the presence or absence of an axon that communicates signals back to photoreceptors (Masland, 2001). However some rodents, including mice, only generate one type of horizontal cell that has an axon for feedback communication (Peichl and González-Soriano, 1994). Interestingly, immunoreactivity profiles indicate no less than four subtypes of horizontal cells in the chicken retina, as have been noted in other avian and reptilian species (Fischer et al., 2007).

Bipolar interneurons are generally referred to as either cone or rod bipolar cells, since they are exclusively connected to one or the other type of photoreceptor. Studies of the anatomy, physiology and gene expression place the number of

different kinds of bipolar cell at twelve (Kim et al., 2008; Masland, 2001b). The main function of bipolar cells varies based on their connectivity, but their two most prominent subdivisions are OFF and ON bipolar cells (Masland, 2012). When photoreceptors depolarize and release glutamate, these cells take up the glutamate and depolarize in turn. Therefore, OFF bipolar cells are active when light is off. ON bipolar cells, on the other hand, are inhibited by glutamate and hyperpolarize in its presence. Only when photoreceptors cease releasing glutamate – that is, when they are hyperpolarized due to the presence of light – do these ON bipolar cells depolarize (Masland, 2012). The ON and OFF subtypes are distinguishable by the location of their dendrites in the inner plexiform layer where the processes meet those of the ganglion cells (Masland, 2001b) – OFF cells do not extend far from the inner nuclear layer (Figure 1B – sublamina A), while ON cells extend farther past their OFF neighbors (Figure 1B – sublamina B).

The most varied class of cells in the retina is the amacrine interneurons. Morphological studies estimate that there are somewhere between 20 and 30 different subtypes of amacrines (depending on the species examined) with an array of receptive fields (MacNeil and Masland, 1998). Unsurprisingly, this morphological diversity correlates with a range of functional roles for amacrine cells, which can be connected to bipolar cells, ganglion cells, and other amacrines in both lateral and direct pathways though the retinal layers (Masland, 1988). These cells are often inhibitory, using the neurotransmitters glycine or γ-aminobutyric acid (GABA). The most common amacrine cell type, named AII, differentially connects rod photoreceptors to the different bipolar cells. After a cluster of rod bipolar cells send

a summative signal to the AII amacrine cell, it uses direct gap junctions to excite ON bipolar cells and chemical inhibition to inhibit OFF bipolar cells, heightening the signal intensity even in low light (Strettoi et al., 1992; Wässle, 2004). Others, such as starburst amacrines, form highly branched and overlapping receptive fields that contribute to directional sensitivity in downstream signaling (Vaney et al., 1988).

1.2.3. Ganglion Cells

Retinal ganglion cells, the main output neurons of the retina, are another diverse group of retinal cells. Their primary purpose is to convey the visual information to the visual centers of the brain (Rockhill et al., 2002). Although this seems a deceptively simple role, the great diversity of retinal ganglion cells has been noted in vertebrates since Ramon y Cajal's studies of the frog retina in 1892, illuminating the true function of ganglion cells not only as a relay station but also as specialized feature detectors of the visual field (Rockhill et al., 2002). Over 20 subtypes of ganglion cells have been isolated with functionality ranging from feature and color detection to direction and motion selectivity photoreception (Rockhill et al., 2002). While some properties of retinal ganglion cell physiology appear to be transiently coded circuits, able to adapt to changing circumstances such as bright or dim light (Grimes et al., 2014), these properties can be distinguished by specific markers, such as the genes JamB or Cdh6, which define distinct subsets of directionally-sensitive OFF retinal ganglion cells (Kay et al., 2011; Kim et al., 2008). Interestingly, these subsets of ganglion cells can be traced throughout retinogenesis as displaying markers that allow them to be identified among other developing

neurons with high accuracy at the very onset of retinogenesis, indicating that their fates may be established transcriptomically early on in development (De la Huerta et al., 2012). Morphological analyses of the ganglion cells present in the chick retina have also resulted in various subset numbers depending on the characteristics used for distinction – for instance, while six main groups of RGCs can be categorized based on their soma sizes and dendritic fields, no less than 26 different stratification patterns exist in the species (Naito and Chen, 2004).

One ganglion cell subtype in particular has been receiving much attention recently. These intrinsically photosensitive retinal ganglion cells (ipRGCs) react to light independently of rods and cones using their own photopigment, melanopsin, and are important in establishing circadian rhythms and the normal pupillary reaction to light (Munch and Kawasaki, 2013). Whereas the primary projection of most feature-detecting retinal ganglion cells is to the lateral geniculate nucleus as a first stop before signals are forwarded to the visual centers in the brain (Field and Chichilnisky, 2007; Wässle, 2004), ipRGC axons project to the suprachiasmatic nucleus and the pretectum, key regulators of involuntary light responses.

1.2.4 Retinal demographics

Each retinal cell type is present in different proportions within the adult retina, although those proportions are conserved within a given species. For example, rods are very common at around 75% of the total retinal cell population in mice, while ganglion cells comprise only 2.5% (Dräger and Olsen, 1981; Young, 1985). It is challenging to develop a full catalog of the subtypes of the most diverse

types of retinal neurons such as amacrine and ganglion cells, especially given the rarity of those cell types in general and their subtypes specifically. Nevertheless, to fully understand the control of retinal cell fate decisions, we must have a better appreciation for the diversity of neuronal end-points. It is only then that we can understand how progenitor cells sort through the myriad cues available to them and decide on a final cell fate. These decisions must be coordinated with the goal of generating the full cohort of retinal cells and functionally distinctive retinal cell subtypes in the right place and time.

2. Retinal cell fate specification

Since the retina is not only the first step in the processing of visual information but also a relatively simple and easily manipulated extension of the central nervous system, it is a widely used model system for studying nervous system development. Optic vesicles first emerge as bilateral evaginations of neuroectoderm from the anterior neural plate (Adler and Canto-Soler, 2007; Fuhrmann, 2010; Martinez-Morales and Wittbrodt, 2009). Upon contacting the surface ectoderm, interactions between the developing tissues leads ectoderm to begin differentiation into the lens placode, while the optic vesicle itself undergoes invagination to form a bilayered optic cup (Fuhrmann, 2010). This cup then continues differentiation into two separate tissues – retinal pigment epithelium and neural retina (Adler and Canto-Soler, 2007).

Once the neural retina is fully developed, the complex processing that is initiated by retinal cells is stunning, and their wide range of responsibilities is only more impressive given the understanding that all this diversity arises from a common progenitor population (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). Each progenitor cell's multivariate fates are guided by a combination of intrinsic signals and differential reactions to extrinsic signals (Livesey and Cepko, 2001). Intrinsic signals include significant differences in gene expression, even among progenitor cells examined at the same developmental time point (Trimarchi et al., 2008). In addition to the intrinsic gene expression of individual progenitor cells, there are also cell-extrinsic factors – signals in the extracellular environment that help push progenitor cells toward a certain cell fate and assist in their path to final differentiation. Studies involving both types of signaling mechanisms are ongoing. Although many important discoveries have been made regarding the control of retinal cell fate decisions, much about the precise combinations of genes and environmental factors and how they interact remains to be discovered. With a greater understanding of the forces driving retinal progenitor cells to a specific neuronal or glial cell fate, we can approach a better understanding of how neurogenesis is accomplished in general.

Though the lessons learned from retinal development can be generalized to other parts of the nervous system, elucidation of how normal retinal development occurs is also important for its own sake. For example, retinal degeneration can specifically or preferentially affect individual subsets of retinal cell types, such as photoreceptors (retinitis pigmentosa or macular degeneration) or ganglion cells

(glaucoma). Though current treatments can ameliorate symptoms and possibly delay degeneration, there are no current interventions that stimulate regeneration of the full functional cohort of lost cells (Kuehn et al., 2005). The only way regeneration could be possible is with a full understanding of the gene expression pathways that drive undifferentiated progenitors to take on those specified roles. With that knowledge, it could be possible to generate the necessary cell types *in vitro* using cultures such as induced pluripotent stem cells (Tucker et al., 2011) or, perhaps, *in vivo* by stimulating the stem cell potential of Muller glia (Karl et al., 2008) .

2.1 Retinal progenitor cells are multipotent

Various approaches have been utilized to uncover the mechanisms through which a progenitor cell chooses its eventual fate during retinal development. One method, called birthdating, stems from the notion that the final division of a progenitor cell is the newly developing daughter cell's "birthday." Birthdating aims to label cells during the DNA synthesis step of the cell cycle by exposing cells to a nucleotide analog such as radioactive thymidine or Bromodeoxyuridine (BrdU) for a period of time (Cepko et al., 1996) . After its uptake during DNA synthesis, the marker becomes diluted by successive cell divisions and strongly marks only those cells that terminally divided immediately after exposure to the marker. Early birthdating studies in the murine retina demonstrated that the different retinal cell types are generated at distinct but overlapping time points throughout development (Sidman, 1961; Young, 1985). The first retinal cells to be generated are the retinal

ganglion cells, followed closely by horizontal cells, amacrine cells, and cone photoreceptors (Farah and Easter, 2005). Later-born cell types include rod photoreceptors, bipolar cells, and Muller glia (Altshuler et al., 1991) (Figure 2A). These birth orders are highly conserved across a multitude of different species, including the chicken (Snow and Robson, 1994; Spence and Robson, 1989). Some recent studies examining amacrine cells more closely have discovered that even some subtypes have distinct birthdays, such that glycinergic amacrine cells are born earlier than their GABAergic relatives (Cherry et al., 2009; Hu and Easter, 1999; Voinescu et al., 2009).

In addition to birthdating, one can track the lineage of a single progenitor cell – that is, all the different cell types it produces – by injecting a developing retina with a low-titer retrovirus expressing a reporter, such as green fluorescent protein (GFP), or other fluorescent dye to allow visualization. Investigations of progenitor lineages demonstrated that retinal progenitor cells are multipotent throughout development, or capable of generating multiple kinds of cells including neurons and glia (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990) (See Figure 2B). These progenitor cells show transient biases in the proportion of cell types they generate, as seen through comparisons of the population of daughter cell types generated at either embryonic or postnatal time points. Specifically, retroviral injections of rat progenitor cells at birth resulted in labeling of rod photoreceptors, bipolar cells, amacrine cells and Muller glia (Turner and Cepko, 1987). When the injections were performed during the embryonic period, many progenitor cells were observed to produce all of the cell types (Turner et al., 1990). Therefore, the

current understanding is that a given progenitor cell is capable of generating several, if not all, of the cell types present in the mature retina. With this knowledge, retinal development studies have focused on elucidating the influence of intrinsic gene expression patterns and the extrinsic environment on each progenitor cells' fate decision.

2.2 Competence in retinal cell fate determination

Based on the data from the lineage experiments, we know that individual retinal progenitor cells are capable of generating an array of cell types. A progenitor cell which is not yet determined to give rise to a particular cell type, but rather has the capability to become any of a variety of retinal cells, is said to have the competence to generate those cell types (Cepko et al., 1996). Retinal progenitor cells are competent to generate a different set of cells at distinct and overlapping timepoints (Figure 2). The probability that a progenitor cell will generate a daughter cell of one type or another changes as development progresses (Figure 2). One example is that early-born retinal cells such as ganglion cells or cone photoreceptors are decreasingly likely to emerge from progenitors of increasingly later stages. The proposed progression of retinal progenitor cells through distinct states of competence is highly reminiscent of the generation of neurons from neuroblast progenitor cells in Drosophila. These neuroblasts produce intermediate cells called ganglion mother cells (GMCs) that in turn produce a stereotyped set of neurons in a distinct order (Pearson and Doe, 2004). Particular transcription factors (*hunchback*, *kruppel*, *PDM* and *castor*) are sequentially activated to control

the transition in neuron production (Pearson and Doe, 2004). Extension in the time that Hunchback is expressed, for example, results in an extension in the generation of the first-born neurons (Isshiki et al., 2001). Misexpression of Ikaros, the mouse ortholog of Hunchback, can prolong the window during which early-born cells are generated, while loss of Ikaros specifically leads to a loss of early-born cells (Elliott et al., 2008). However, these effects were not as pronounced as those induced by *hunchback*. Interestingly, a converse experiment misexpressing and conditionally deleting Castor (Casz1), a gene that is present in mid- and late-stage RPCs and is repressed by the appropriate Hunchback homologue in both Drosophila and mouse, found that mouse Casz1 is both necessary and sufficient to inhibit generation of early-born cells (Mattar et al., 2015). Currently it is unclear exactly which upstream or downstream factors control these changes in retinal neuron production over time.

The likelihood of a progenitor cell producing one cell type over another could be determined by intrinsic factors (the genes expressed by that progenitor cell), extrinsic factors (signals contributed by the progenitor's environment), or some combination of the two. The extent to which intrinsic and extrinsic signals influence a progenitor cell's fate decision is still a hotly debated topic. Several studies have attempted to address the relative importance of these signals. In the first study, embryonic retinal progenitor cells were isolated, mixed with an excess of postnatal cells and their resulting cell fates analyzed. Importantly, no embryonically derived progenitor cells were observed to prematurely adopt any postnatally generated cell fate (Belliveau and Cepko, 1999). The same basic idea held true during the converse

experiment. Progenitor cells derived from postnatal retinas did not adopt early-generated cell fates when mixed with an excess of embryonic cells (Belliveau et al., 2000). While there were slight changes in the relative proportions of some cells, these experiments point to intrinsic signaling as the driving force behind a progenitor cell's ability to become a given cell type, in spite of any environmental signals. In one final experiment, rat progenitor cells that were dissociated from one another and cultured in vitro generated the normal proportions of the different retinal cells with the correct timing (Cayouette et al., 2003). Again, these results demonstrate that retinal cell fate decisions proceed normally even in the absence of normal extracellular signals.

2.3 Models of retinal cell fate determination

A discussion of the influences that different intrinsic and extrinsic factors can have on the acquisition of different retinal cell fates suggests that retinal progenitor cells are enacting discrete genetic programs that lead to the reproducible production of particular types of cells. These programs are represented in the deterministic model of cell fate determination (Figure 3A). Perhaps the most well-known example of such a deterministic organization of cell fate occurs in C. elegans. Researchers have discovered the identity and number of all the cells in this nematode and even have mapped out the lineages for all cells, as they do not change from one animal to another (Hobert, 2010). While the case of C. elegans is an extreme one, it has been presumed for some time that cell fate determination generally follows the same basic rules. In the vertebrate retina, this would be that

cells enact a specific intrinsic program, at a specific time and specific position. The results of that intrinsic cell fate program would be the production of a specific cell type. This program would be the same each time this particular retinal neuron type was produced. As development proceeds, the competence of the progenitor cells would change, but the intrinsic programs they enact would be the same for any particular cell type. However, more recent data suggests that not all the cell fate decisions of retinal progenitor cells are deterministic, but instead there may be more randomness to these decisions than was previously appreciated (Figure 3B).

Stochastic cell fate determination has been demonstrated in Drosophila eye development, where progenitors are randomly recruited to clusters of eight photoreceptors, or ommatidia, and adopt a photoreceptor fate based on their position and timing of recruitment. Extracellular Notch/Delta signaling plays a critical role in the differentiation of cells in the ommatidia. Progenitor cells are initially indistinguishable until small changes in the cells' reaction to Notch signals initiate feedback loops to differentiate those cells further (Johnston and Desplan, 2008). Removal of the sevenup gene, which is repressed by Notch and is necessary to generate R1 and R6 photoreceptors, prevents progenitor cells from developing properly, and leaves them to decide randomly between an R7 or an R8 photoreceptor fate (Miller et al., 2008). The cells forced into these fates decide to become either R7 or R8 regardless of spatial considerations, and express genes specific to one photoreceptor type completely exclusively of the other (Miller et al., 2008).

The gene networks involved in the cell fate decision-making process in vertebrates are complex and dynamic, which impedes experiments designed to differentiate between a stochastic and deterministic model. To begin to gain insight into which process is operating, researchers first set out to understand the types of divisions that progenitor cells undergo. These divisions can be classified as proliferative (generating two new progenitors), self-renewing (generating one progenitor cell and one differentiated retinal cell), and terminal (generating two non-proliferative retinal cells). In zebrafish, marked lineages of progenitor cells were monitored through multiple cell divisions and the resulting data used to generate a mathematical model of retinal development. The best model to explain the data was one where the progenitor cells produced progeny without adherence to a strictly deterministic pattern (He et al., 2012). The model is not consistent with a fully stochastic model either, but rather one in which progenitor cells make "random" cell fate choices based on a weighted probability that changes throughout development (Figure 3). Interestingly, this study also showed that the bHLH factor ath5 can "tip the scales" in favor of ganglion cell production, linking a factor with a wealth of previous literature to this new model of retinal development (He et al., 2012). This probabilistic mode of cell fate determination is not unique to zebrafish. When videomicroscopy was used to monitor rat progenitor cells and the cell fates they produce over time, it was found that again the most consistent model of cell fate acquisition was a more stochastic one (Gomes et al., 2011). Specifically, a progenitor cell was able to generate a late-born neuron and then an early-born retinal neuron in succession (Gomes et al., 2011). These observations suggest a

stochastic probability that each progenitor cell could differentiate into any of a few given cell types, as opposed to a more deterministic timed cell lineage.

3. "Omics" approaches to understanding retinal cell fate decisions

3.1 Whole retina studies

Classic studies designed to identify and characterize retinal cell fate determinants traditionally focused on the role of a single factor or, in rare instances, several factors simultaneously. However, it is undoubtedly combinations of genes (networks) that work together in concert to produce the distinct types and subtypes of retinal neurons. Therefore, in the search for intrinsic and extrinsic factors that work together to drive the fate of retinal progenitor cells, it is important to leave no stone unturned. Forward genetics, the technique by which an interesting phenotype (such as microphthalmia or lack of optic nerve development) is discovered and studied to determine its genetic root, is an extremely powerful approach to determine genes that drastically affect large-scale developmental cascades. However, many genes may be missed by forward genetics. A converse approach, called reverse genetics, starts with a gene in mind and works backward to determine the role that gene plays in a system. The process of reverse genetics may seem less exciting at first, but it allows for a much more thorough understanding of developmental factors. The key to a reverse genetics approach is knowing the transcriptome – the full collection of mRNA transcripts that are expressed, whether in the whole retina, a subset of retinal cells, or just within a single developing cell.

Many recent studies have tackled the problem of identifying all the mRNA transcripts associated with different stages of retinal development and distinct retinal cell populations.

Several groups have examined retinal gene expression using microarrays or expressed sequence tag (EST) based approaches to catalog all the transcripts expressed at distinct times during normal retinal development (Chowers et al., 2003; Livesey et al., 2004; Swaroop and Zack, 2002). Additionally, comparisons of transcriptomes between wildtype and mutant organisms can help to reveal previously uncharacterized factors involved in the development of particular retinal cells. For instance, one gene with particular importance to RGC genesis is Brn3b. In the absence of Brn3b,RGC populations were decreased by 80% (Gan et al., 1996). A screen for genes differentially expressed between embryonic day (E)14.5 wildtype and Brn3b knockout mice revealed a host of candidate genes to be tested for their functional roles in ganglion cell development (Mu et al., 2001). Comparisons have also been made between the transcriptomes of wildtype mice and Math5-deficient mice at multiple developmental time points. These microarray experiments revealed a wealth of potential downstream targets of Math5 that may be important in the cell fate determination of several early-generated retinal cell types (Mu et al., 2005). As with all of these large-scale profiling studies, the future challenge is to understand which of these genes are the critical cell fate regulators and how all of these different genes work together to produce the different retinal cells.

To further expand our knowledge of gene expression, serial analysis of gene expression, or SAGE, was employed at two day intervals throughout retinal development (Blackshaw et al., 2004). This technique has the added advantage of not relying solely on those genes that are spotted down on a microarray. Additionally, this study further characterized the expression of >1000 genes by *in situ* hybridization (Blackshaw et al., 2004). Mining this dataset has already identified transcription factors and signaling molecules that could play key roles in setting up distinct cell fate choices. As sequencing techniques have improved, RNA-seq has now become a widely used profiling technique. Thus far it has only been used to identify mRNAs expressed in the adult retina (Gamsiz et al., 2012), but it will soon be utilized for multiple different time points. RNA-seq allows for the identification of novel splice isoforms and long non-coding RNAs to add to the repertoire of potential cell fate determinants (Wang et al., 2009) . These studies will continue to produce candidate factors for study for many years to come.

3.2 Single cell studies

Since the developing retina contains a complex mixture of uncommitted retinal progenitor cells and different cell types in various stages of fate determination and maturation (Young, 1985), identifying programs that drive distinct cell fates is challenging with classic whole tissue approaches. Given these issues, single cell-based profiling techniques were adapted to retinal cells to achieve the resolution necessary to examine gene networks in even rare cell types. After retinal dissection and dissociation, individual cells were isolated using a small glass

needle and cDNA libraries were generated through amplification-based strategies (Goetz and Trimarchi, 2012). The resulting microarray data from these single cell libraries revealed a treasure trove of marker genes for mouse retinal progenitor cells at different stages of development (Trimarchi et al., 2008) and, specifically, for developing retinal ganglion and amacrine cells (Trimarchi et al., 2007). In addition to the new markers, the single cell retinal profiling studies revealed a surprising level of gene expression heterogeneity, even among retinal progenitor cells isolated from the same embryonic time point (Trimarchi et al., 2008). Much of this observed heterogeneity was observed to derive from the differential expression of transcription factors from diverse sets of transcription factor families, including those described in this review and many others. Despite this wealth of expression-based information, precisely how these genes work together to produce each specific type and subtype of retinal neuron is not fully understood. A combination of sophisticated computational tools and manually-driven functional studies will be necessary to generate a more complete picture of the role(s) for each of these genes in retinal cell fate determination.

Since it was known that retinal progenitor cells change their competence as they progress through developmental time (Cepko et al., 1996) and that this progression appeared to be largely controlled by intrinsic mechanisms (Cayouette et al., 2003), one would predict that clusters of genes would track specifically with either early or late single retinal progenitor cells. While this was indeed found to be the case, the number of genes and their expression patterns proved to be a surprise. Only a single gene, Secreted frizzled-related protein 2 (Sfrp2), was found to be

broadly expressed primarily in early embryonic progenitor cells. A handful of additional genes showed expression that was restricted to early progenitor cells, but each of these genes was only observed in a small subset of cells. What these results mean in terms of the competence model of retinal development remains unclear. Perhaps competence is not driven by changes in mRNAs expressed in progenitor cells, but rather by changes in protein expression (see Ikaros), changes in miRNA expression (Smirnova et al., 2005), or some combination of both. Alternatively, the gene expression heterogeneity revealed by the single cell profiling could be pointing to a more stochastic model of retinal development. No matter which model proves to be ultimately correct, collecting more single cell profiles will help to sort through the meaning of all the gene expression heterogeneity. For these future studies either genetically encoded or electroporated fluorescent reporters (Cherry et al., 2009; Matsuda and Cepko, 2007) will enable the isolation of more specific sets of cells. This refinement, combined with the change from microarray technology to RNA-seq technology, will expand the amount of information collected and the number of single cells analyzed.

4. Intrinsic signals and retinal development

In addition to external signaling molecules, intrinsic factors expressed within a given retinal progenitor cell can play critical roles in determining final cell fate decisions. Most often, these intrinsically-expressed proteins are transcription factors that bind specific DNA sequences and control the expression of cohorts of downstream genes that execute the cellular differentiation program. Given that my

work has focused on the cell-intrinsic mechanisms driving cell fate decisions, this introduction will primarily examine a few of the myriad transcription factors that have been shown to influence retinal cell fate and then discuss the roles of more recently identified cell fate effectors.

4.1 Basic helix-loop-helix factors

Members of the basic helix-loop-helix (bHLH) transcription factor family have been shown to be involved in multiple developmental processes, including retinal neurogenesis (Lee, 1997). These proteins are composed of a helix-loop-helix domain that contributes to dimerization with other bHLH factors, and a basic region that binds to the E-box sequence (CANNTG) of DNA (Murre et al., 1989). In addition, different bHLH proteins can either repress or activate the transcription of downstream genes. Generally the bHLH activators promote neurogenesis, while the repressors work to inhibit neuronal differentiation (Hatakeyama and Kageyama, 2004). Given that different combinations of all the bHLH factors are expressed in different subsets of retinal progenitors (Brzezinski et al., 2011; Trimarchi et al., 2008), their contributions to individual retinal cell fates have proven to be a bit more complicated than was initially predicted.

4.1.1 Ath5

Ath5 belongs to a subfamily of bHLH transcription factors that is homologous to the Drosophila atonal protein. Atonal is required for the specification of the initial photoreceptor (R8) during Drosophila eye development (Jarman et al., 1994,

1995). Given this result, it was of great interest to test whether vertebrate homologs of *atonal* could play similar roles. Overexpression of the Xenopus homolog, Xath5, significantly increased the population of retinal ganglion cells and led to fewer amacrine cells, bipolar cells and Muller glia when injected into the frog (Kanekar et al., 1997). This effect appeared specific to Xath5 as overexpression of a different bHLH factor, NeuroD, did not lead to a change in ganglion cell numbers, but rather to an increase in amacrine and bipolar cells (Kanekar et al., 1997). However, it is unclear whether this effect of Xath5 is generalizable or species specific. Overexpression of the mouse homolog, Math5, in the same assay resulted in an increase in bipolar cells and not an increase in ganglion cells (Brown et al., 1998). Using viruses to misexpress either Math5 or the chicken homolog (Cath5) in developing chicken retinas did lead to an increase in the number of cells expressing ganglion markers (Liu et al., 2001). In total, misexpression of Ath5 in different organisms pointed toward a role in retinal cell fate acquisition with a specific emphasis on retinal ganglion cells. The exact nature of that cell fate role was not fully clear as homologs from different species displayed different potencies.

To better understand the role of Math5 in cell fate determination, it helps to know exactly which cells in the mature retina have a history of Math5 expression. To trace the lineage of Math5 positive progenitor cells, cre recombinase was inserted into the Math5 locus and these mice were crossed to a cre-dependent reporter mouse for cell visualization. These mice demonstrated that the lineage of Math5-expressing cells includes most of the ganglion cells and some photoreceptors, amacrine cells, and horizontal cells (Yang et al., 2003). A second cross of the Math5-

cre mice to a GFP reporter confirmed the earlier lineage results and showed that Math5-expressing cells contribute to both rod and cone photoreceptor populations and to a subset of amacrine cells (GABAergic, cholinergic and AII subtypes)(Feng et al., 2010). Given the multitude of cell types with a history of Math5 expression, a simple model where Math5 instructively drives the formation of ganglion cells seems unlikely.

The role of Math5 in retinal cell fate determination was further investigated through the generation of Math5-deficient mice by several laboratories. In the absence of Math5, many cell fate-related phenotypes were observed including some that differed between the mice from distinct labs. The most consistent phenotype was a severe reduction in ganglion cell populations (80-90% of ganglion cells lost) (Brown et al., 2001; Wang, 2001). In fact, complete loss of ganglion cells is also observed in the Lakritz zebrafish mutant for Ath5 (Kay et al., 2001). However, additional scrutiny revealed other retinal phenotypes in the Math5 knockout mice. In at least one study, the number of cone photoreceptors increased (Brown et al., 2001). This observation suggested a possible simple cell fate switch from ganglion cells with Math5 to cone photoreceptors without Math5. The situation has proven to be more complicated than that simple model, though, as the amacrine cell population is also affected in the absence of Math5. One study found that syntaxin, a marker of all amacrine cells, was expressed normally in the absence of Math5 (Brown et al., 2001). Even with this normal expression, however, the numbers of two subtypes of amacrine cells, AII and dopaminergic, were substantially decreased in Math5 mutant mice (Brown et al., 2001). Surprisingly, in a different study with a

different Math5 mutant mouse, syntaxin staining was increased and cholinergic amacrine cells (as defined by ChAT staining) were also increased (Wang, 2001). Altogether the results from the Math5-deficient mice demonstrate the importance of this bHLH factor in cell fate acquisition in the early developing retina.

Recent Math5-related studies have focused on deciphering the complexity of bHLH interactions and compensation. When Math5-null mice instead express the related bHLH factors NeuroD1 or Math3 under the Math5 promoter, retinal ganglion cell development is partially rescued to 40% and 10% of wildtype populations, respectively (Mao et al., 2008). Conversely, insertion of Math5 into the NeuroD1 locus reprogrammed future amacrine cells into retinal ganglion cells (Mao et al., 2013). However, this is not a universal feature of Math5 since it could not convert rod photoreceptor precursor cells into ganglion cells when inserted into the Crx gene locus (Prasov and Glaser, 2012). All bHLH factors were not created equal, as replacement of Math5 with a different bHLH, Ascl1, failed to rescue ganglion cell production (Hufnagel et al., 2013). These results demonstrate that bHLH factors including Ath5 execute their functions in a context dependent manner. Under certain conditions, Ath5 expression provides a permissive environment for the production of ganglion cells, whereas under other conditions it fails to do so. Unraveling the additional factors and precise conditions are challenges for future research.

4.1.2 NeuroD family

The NeuroD family of bHLH transcription factors is also important in eye development; the various family members have a range of overlapping, though not identical, expression patterns and effects. Overexpression of NeuroD1 leads to an increase in rod photoreceptors at the expense of Muller glia (Morrow et al., 1999; Ochocinska and Hitchcock, 2009). However, mice engineered to lack NeuroD1 only show a slight increase in bipolar cells (Morrow et al., 1999). Amacrine cell differentiation is delayed, but by adulthood the total number of amacrine cells is normal in NeuroD1 deficient mice (Morrow et al., 1999). Generating a compound mutant mouse where both NeuroD1 and another bHLH factor, Math3, are deleted resulted in a complete loss of amacrine cells and an increase in both ganglion cells and Muller glia (Inoue et al., 2002). Overexpression experiments, meanwhile, have demonstrated that while these bHLH factors are necessary for amacrine cell fates, they are not sufficient (Inoue et al., 2002). Other factors, either bHLH factors or factors belonging to additional transcription factor families, are clearly necessary to combine with these bHLHs to drive the cell fate determination of amacrine cells. In an interesting twist on these overexpression experiments, NeuroD1 was recently knocked into the Math5 locus in mice. This meant that there was no endogenous Math5 present and NeuroD1 was now expressed under the control of Math5 regulatory sequences. When this is the case, NeuroD1 is capable of partially rescuing the production of ganglion cells in a Math5-deficient mouse (Mao et al., 2008). These results show that while NeuroD1 has evolved its own functions in amacrine and photoreceptor cell fate specification, it still has the capacity to behave

similarly as a completely distinct bHLH factor when expressed at a different time and place.

Recently, other NeuroD family members also have been implicated in retinal cell fate determination. Loss of NeuroD2 in mice led to a specific decrease in AII amacrine cells while overexpression led to an increase in amacrine cells at the expense of bipolar cells and Muller glia (Cherry et al., 2011). Surprisingly, overexpression of NeuroD2 at postnatal day 0 (P0) led to an increase in the production of ganglion cells beyond the time when retinal progenitor cells are normally competent to produce these cells (Cherry et al., 2011). Removal of another related family member, NeuroD6, also leads to a change in amacrine cell fate. In this case, more glycinergic amacrine cells are produced at the expense of GABAergic cells (Kay et al., 2011) . These experiments show that to fully understand the generation of all retinal cells, studies of cell fate determination must be brought to the level of small subtypes of retinal neurons.

4.1.3 Acsl1/Mash1

Acsl1, also referred to as Mash1, is the mammalian homolog of the Drosophila achaete-scute like family of bHLH transcription factors. Mice deficient for Acsl1 die at birth, making a thorough assessment of early and late-generated retinal fates *in vivo* impossible without use of a conditional knockout mouse (Guillemot et al., 1993). Experiments in which retinas were explanted and cultured *ex vivo* revealed a possible cell fate connection between Ascl1 and bipolar cells (Tomita et al., 1996). Retinas that were mutant for both Ascl1 and another bHLH

factor Math3 displayed a complete loss of bipolar cells and an increase in Muller glia in explant culture experiments (Tomita et al., 2000). Taken together, these mouse results point to a role for Ascl1 in bipolar cell fate determination, but since *in vitro* culture experiments can be difficult to interpret in the absence of the *in vivo* environment, further experimentation is warranted. Recently, some interesting results have come to light regarding Ascl1 and its ability to promote neurogenesis at the expense of gliogenesis. In the zebrafish, *ascl1a* is upregulated rapidly after retina injury and is required for retinal regeneration (Fausett et al., 2008). In mouse Muller glia, forced expression of Ascl1 in Muller glia led to a downregulation of glial gene expression and an acquisition of the capacity to produce retinal neurons (Pollak et al., 2013) . These observations reinforce the idea that insights gleaned from retinal cell fate experiments will be crucial for informing efforts to achieve regeneration of particular retinal cells or entire retinas.

To gain further insight into the role of Acsl1 in retinal cell fate acquisition, lineage tracing studies were performed to determine which cells had a history of Acsl1 expression. In general, all major cell classes in the retina were represented except for the nearly complete lack of retinal ganglion cells derived from Acsl1-positive progenitors (Brzezinski et al., 2011). Additionally, the other early-generated retinal neurons (horizontal cell, cone photoreceptors and amacrine cells) were over-represented in the Acsl1-lineage, while rod photoreceptors were under-represented (Brzezinski et al., 2011). These results indicate that the heterogeneity in transcription factor expression observed in retinal progenitor cells can affect the competence of particular cells to choose a certain cell fate.

4.1.4 Additional bHLH family members

Many other bHLH transcription factors are also expressed in the developing retina (Blackshaw et al., 2004; Trimarchi et al., 2008) and are likely involved in retinal cell fate determination. For instance, deletion of Bhlhb5 in the mouse results in a decrease in GABAergic amacrine cells and a subset of bipolar cells (Feng et al., 2006). Olig2, another bHLH from the Olig subfamily, is expressed in only a small subset of retinal progenitor cells (Hafler et al., 2012). Cone photoreceptors, horizontal cells, amacrine cells and bipolar cells all displayed a history of Olig2 expression. Overexpression of Olig2, however, did not convey any specific cell fate, but rather led to a general cell cycle exit (Hafler et al., 2012). In addition, loss of Olig2 did not lead to any observable cell fate defect (Hafler et al., 2012). These results point to the fact that distinct bHLH factors have distinct roles in retinal cell fate determination. Further studies will be required to fully elucidate any overlapping and combinatorial roles these transcription factors might play.

Pancreas transcription factor 1a (Ptf1a) is a bHLH factor that had been linked to cell fate acquisition in the developing pancreas (Kawaguchi et al., 2002). Lineage tracing analyses in the retina showed that only horizontal and amacrine cells have a history of expressing Ptf1a (Fujitani et al., 2006; Nakhai et al., 2007). Loss of Ptf1a in the developing mouse retina led to a complete loss of horizontal cells, a significant decrease in amacrine cells and a corresponding increase in ganglion cells (Fujitani et al., 2006; Nakhai et al., 2007). Along these same lines, overexpression of Xenopus Ptf1a led to an increase of GABAergic cell types, such as horizontal and amacrine

cells (Dullin et al., 2007). Ptf1a plays a similar role in the developing mouse retina, although while coincident misexpression of Ptf1a and the homeodomain transcription factor Onecut1 is necessary and sufficient to promote a horizontal cell fate, neither gene alone appears to be sufficient to generate ectopic horizontal cells (Wu et al., 2013). These experiments demonstrate that this bHLH factor is a key player in the cell fate determination of horizontal and amacrine cells.

4.2 Homeodomain-containing transcription factors

Homeotic genes are perhaps most well-known for the Drosophila mutants where one body segment has transformed into another such that the mutant flies can have a pair of legs emanating from their head (Gehring and Hiromi, 1986). However, homeodomain-containing proteins can regulate many different developmental processes including cell fate determination. These proteins contain a widely conserved homeobox domain that is 180 bp in length and is responsible for conferring sequence specific DNA binding activity on this set of transcriptional regulators (Gehring et al., 1994). Homeodomain-containing transcription factors are well known for their importance in the initial formation of the eye field itself (Zuber et al., 2003), but I will not discuss these roles in detail here. Not surprisingly, many homeodomain transcription factors are involved in the determination of particular retinal fates and I will discuss several examples here to provide a framework for understanding the functions of this family.

4.2.1 Vsx2/ Chx10

Visual system homeobox 2 (Vsx2), formerly known as Chx10, is one member of the homeodomain transcription factor family. Mice that harbor a mutation in the Vsx2 gene display microphthalmia (small eye) (Burmeister et al., 1996), primarily due to the upregulation of a cyclin-dependent kinase inhibitor, $p27^{Kip1}$, in retinal progenitor cells (Green et al., 2003) . In fact, removal of $p27^{Kip1}$ in a Vsx2 mutant mouse significantly rescues the small eye phenotype by restoring progenitor cell proliferation (Green et al., 2003). However, these mice also showed that Vsx2 is critically important for the production of bipolar cells, as they are absent in both Vsx2 mouse models (Burmeister et al., 1996; Green et al., 2003). Vsx2 is not simply instructive for bipolar cell production on its own. This is observed through Vsx2 overexpression experiments that led to an increase in Muller glia cells (Hatakeyama et al., 2001). However, when Vsx2 was expressed in combination with either of two bHLH proteins, Math3 or Mash1, an increase in bipolar interneurons was observed (Hatakeyama et al., 2001). These experiments demonstrate that homeodomain proteins play important roles in specific retinal cell fates and that combinations of transcription factors from different families will prove critical for driving cell fate acquisition in different retinal cell types.

4.2.2 Otx2

A second homeodomain transcription factor expressed during retinal development is Otx2 (Baas et al., 2000). Conditional removal of mouse Otx2 in the retina leads to a complete loss of photoreceptor and bipolar cells with a

corresponding increase in several different types of amacrine cells (Koike et al., 2007; Nishida et al., 2003). Misexpression of Otx2 in P0 rat retinas promotes a photoreceptor cell fate at the expense of the other late-generated cells (amacrine, bipolar and Muller glia) (Nishida et al., 2003). These results point to Otx2 as a key inducer of the photoreceptor fate and, perhaps, as a repressor of the amacrine fate. It is noteworthy that a highly related transcription factor, Crx, is not involved in photoreceptor cell fate, but rather in the further downstream terminal differentiation of photoreceptors (Chen et al., 1997; Furukawa et al., 1997)(Chen et al., 1997; Furukawa et al., 1997). This indicates that there is an exquisite specificity operating at the level of these cell fate-inducing transcription factors. In fact, it has recently been demonstrated that another homeodomain transcription factor, Rax, can activate the Otx2 promoter, while the Notch pathway has been implicated in the repression of Otx2 (Muranishi et al., 2011). Deciphering the interplay of these intrinsic mechanisms and extrinsic signaling pathways is an important future direction for gaining a complete understanding of how retinal cell fate determination is controlled.

4.2.3 Rax

Retinal homeobox 1 (Rax) is a homeodomain-containing transcription factor that is essential for the development of the eye (Mathers et al., 1997). Retroviral misexpression of Rax resulted in an inhibition of neurogenesis and a promotion of Muller glia formation (Furukawa et al., 2000). This glial induction may be occurring through a Notch1 dependent mechanism (Furukawa et al., 2000). However, it is still

not known what role endogenous Rax is playing in retinal cell fate determination, since removal of murine Rax results in a complete loss of eyes. A fuller understanding of the role of Rax in cell fate determination awaits the characterization of cell-type specific knockout mice. One potential hint as to the role of Rax in individual cell types comes from studies on the zebrafish homologues. Zebrafish contain three Rax-related genes (rx1, rx2 and rx3). Morpholino mediated knockdown of either rx1 or rx2 during the period of photoreceptor production led to a decrease in many photoreceptor-expressed mRNAs (Nelson et al., 2009). Through the use of these "late-injected morphants" these studies were able to reveal a possible role for rx genes in the acquisition or maintenance of the photoreceptor fate.

4.2.4 Pax6

The paired-type homeobox transcription factor Pax6 has powerful functions in eye development. It is essential for the development of the eye field and, when overexpressed in Drosophila, generates ectopic eyes on wings, legs and antennae (Grindley et al., 1995; Halder et al., 1995; Nornes et al., 1998). To bypass the early requirement for Pax6 and to better understand its downstream cell fate effects, the gene was conditionally inactivated in older retinal progenitor cells. In the absence of Pax6, progenitor cells surprisingly produced only amacrine interneurons (Marquardt et al., 2001). The mechanism by which Pax6 normally controls the multipotency of progenitor cells is still unclear, but it most likely involves the regulation of a network of other transcription factors. For example, Pax6 has been

show to positively regulate the bHLH factor Math5 and negatively regulate the photoreceptor transcription factor Crx (Oron-Karni et al., 2008; Riesenberg et al., 2009). It will be important, as part of future studies, to fully define the gene networks downstream of Pax6 and to link each of those networks to specific functions of this important transcription factor.

4.2.5 Other homeodomain transcription factors

Other homeodomain-containing transcription factors also play significant roles in retinal cell fate acquisition. When both of the Distal-less homeobox proteins Dlx1/Dlx2 are removed from the retina, increased apoptosis accompanied a marked thinning of the RGC layer (de Melo et al., 2005). These double-knockout mice are embryonic lethal so it is difficult to discern the precise downstream targets of the Dlxs and whether they play any additional roles in other cell types. Postnatal misexpression of the homeodomain transcription factor Prox1 led to an increase in horizontal cells (Dyer, 2003). Along the same lines, deletion of mouse Prox1 led to the complete absence of horizontal cells demonstrating that this factor is both necessary and sufficient for the production of horizontal cells (Dyer, 2003). These experiments also showed a potential role for Prox1 in amacrine cells, rod photoreceptors and Muller glia (Dyer, 2003). As with all the other factors involved in retinal cell determination, it will be of interest to delineate the exact downstream targets that are involved in the cell fate controls of the different retinal cell types.

4.3 Other transcription factors and their effects on retinal cell fate

While the bHLH and HD families are two of the largest with identified roles in retinal cell fate determination, there are many additional intrinsic factors that also influence retinal cell fate. Many members of the forkhead/winged helix family of transcription factors are expressed in the developing retina (Trimarchi et al., 2008). Mice deficient for one of these members, Foxn4, show a near complete loss of amacrine cells, a complete ablation of horizontal cells and an increase in photoreceptor cells (Li et al., 2004). Misexpression of Foxn4 results in the over-production of amacrine cells, showing that Foxn4 is both necessary and sufficient for this retinal cell fate (Li et al., 2004). The downstream mechanisms through which Foxn4 exerts its effects are still being worked out, but this factor has been shown to impact numerous pathways, including upregulating bHLH and HD transcription factors and influencing Notch activity through the regulation of a Delta (Dll4) (Li et al., 2004; Luo et al., 2012). Given the plethora of Forkhead factors expressed during retinal development, it will be of interest to determine the phenotypes of all the others, alone or in combination.

Undoubtedly this introduction, as well as the wide range of studies not covered herein, has only scratched the surface in our understanding of the roles played by different transcription factors and their downstream networks in retinal cell fate determination. While I have highlighted some of the larger transcription factor families in this review, there are certainly additional factors that have been omitted. Each year, phenotypes related to additional factors are identified and

added to the growing knowledge of the cell fate process. In the future, the biggest challenge will be to integrate all of these distinct factors and their regulatory networks into a comprehensive snapshot of how the cell fate programs are initiated and executed to produce not only each retinal cell type, but their subtypes as well. With this information in hand, researchers will be better able to produce or regenerate any retinal cell types that may be dying or dysfunctional in different retinal disorders.

5. Other influences on cell fate determination

5.1 Asymmetric versus symmetric cell divisions

We have discussed many intrinsic and extrinsic factors and the roles they play in retinal cell fate decisions, but there are other aspects to the story. For example, the position and activity of a progenitor cell during its progression through the cell cycle may confer information about its final cell fate beyond its internal composition and the nearby environmental factors. It is well documented that the nuclei of cycling progenitor cells follow a predictable progression (Dyer and Cepko, 2001a). They migrate to the apical, or outer, surface of the retina to undergo mitosis. As they move forward through the first gap (G1) phase, these progenitor cells shift basally, toward the vitreal surface of the retina. At their basal nadir, progenitor cells pass through G1 and start the S phase of the cell cycle. They then move apically again in G2 phase before entering mitosis again at their apical peak. By studying the kinetic trends of progenitor cells, it was found that cells that travel much farther

basally than their neighbors tend to generate two neurons instead of another proliferating progenitor cell (Baye and Link, 2007). However, whether this phenomenon is linked to specific retinal fates or just neurogenesis in general remains to be determined.

In Drosophila and *C. elegans*, asymmetric distribution of cell fate determinants prior to cell division is widely known to bias the cell fate decision-making process (Roegiers and Jan, 2004). Uneven distribution of mRNAs or proteins within a dividing parent cell can produce different fates between its two daughter cells. One identified factor that is asymmetrically localized at mitosis is Numb, a negative regulator of Notch. When Numb is misexpressed in a progenitor cell, it has a higher chance of producing rod photoreceptors at the expense of amacrine cells, bipolar cells and Muller glia (Cayouette and Raff, 2003). Since Numb inhibits Notch signaling, these results are consistent with those where Notch itself was perturbed. Loss of Notch1 late in retinal development led to an increase in rod photoreceptors much like overexpression of Numb. Removal of Numb from the retina leads to a corresponding decrease in asymmetric cell divisions that produce a photoreceptor cell. However, symmetric divisions producing rod photoreceptors are increased, indicating that Numb is not generally required for this particular cell fate (Kechad et al., 2012). What are the determinants that drive rod photoreceptor fate in symmetric divisions versus asymmetric ones? Why does there need to be a difference in how these cells acquire their fates? The answers to these questions await further experimentation.

5.2 The cell cycle's influence on cell fate

Underlying all of the stimuli (extrinsic and intrinsic) that influence retinal cell fate is the fact that this process must be coordinated with cell proliferation so that the retina produces the correct amount of total cells. Many extrinsic and intrinsic factors that we have discussed influence the cell cycle in addition to cell fate. Cell cycle regulators also aid in the cell fate decision-making process and perhaps, in some cases, help drive the specification of particular fates. One well-studied factor that is critically involved in the regulation of cell cycle exit is the retinoblastoma tumor suppressor protein (Rb) (Weinberg, 1995). Conditional knockout mice where Rb is deleted from the retina do in fact show a defect in the ability of progenitor cells to exit the cell cycle (Zhang et al., 2004). Interestingly, loss of Rb also leads to the production of fewer rod photoreceptors specifically (Zhang et al., 2004). This cell fate phenotype occurs only in rod photoreceptors and is separate from the effect of Rb loss on the cell cycle. Instead, transcription factors that are important for rod development, such as Nrl, are absent in Rb deficient retinas (Zhang et al., 2004). These results demonstrate that factors can play different roles in determining different cell fates and that, for some factors, their cell cycle roles are separate from other roles in cell fate determination.

Another family of cell cycle regulators, the cyclin kinase inhibitors (CKIs), can block the cell cycle at a number of places and, in some contexts, promote differentiation. Overexpression of a CKI from Xenopus, p27Xic1, inhibits retinal progenitor cell proliferation, but also shows a specific cell fate effect. Muller glia

cells are increased, while bipolar cells are decreased (Ohnuma et al., 1999). Interestingly, the domain that is responsible for kinase inhibition was not required to facilitate the cell fate switch, suggesting a potential novel mechanism (Ohnuma et al., 1999). Both gain and loss of the mouse homolog of this CKI, p27Kip1, indicate cell cycle phenotypes in a similar fashion as p27Xic1. However, in the case of the mouse, neither gain nor loss of function showed significant alterations in any retinal cell fates (Dyer and Cepko, 2001b). Perturbation of another related mouse CKI, p57Kip2, displays aspects of both a cell cycle and cell fate phenotype, much as in the case of Xenopus. When p57Kip2 was removed from embryonic retinal progenitor cells, these cells could not properly exit the cell cycle and subsequently died (Dyer and Cepko, 2000). On the other hand, when p57Kip2 was removed from postnatal retinal progenitor cells, there was an increase in amacrine cells (Dyer and Cepko, 2000). All of these experiments point to the precise regulation of intrinsic factors (transcription factors and otherwise) in response to extrinsic cues leading to a coordinated exit from the cell cycle and the acquisition of specific retinal cell fates. It is important to consider how all of these processes are co-regulated when examining any experimental results dealing with cell fate determination.

6. Conclusions

As we move to the future, how are we to make sense of the strategies retinal progenitor cells use to acquire all the distinct cell fates (both cell types and subtypes)? It is apparent that a simple model wherein cells proceed along an intrinsically determined linear pathway and arrive at a specific fate is insufficient. It

is here that single cell gene expression profiling studies will be invaluable for revealing the gene networks operating to push cells down one cell-fate pathway or another. Experiments performed on individual developing retinal cells have already demonstrated an incredible amount of gene expression heterogeneity in the cycling cell population (Trimarchi et al., 2008). Nowhere is this more apparent than when examining different subsets of transcription factors. The challenge for the future will be to determine just which combinations of factors are the critical ones for biasing a progenitor cell toward one fate or another. Although it is possible to imagine many different approaches to better understand the combinations of intrinsic factors that drive cell fate decisions, two main approaches are outlined for the remainder of this dissertation. First, the information gleaned from transcriptomes of individual retinal cells has provided unprecedented resolution to study the heterogeneity of retinal progenitors. Specific genes have been identified from these transcriptomes for functional studies. First, the Onecut family of transcription factors was observed in subsets of Math5+ retinal progenitors, and through characterization of Onecut1 and Onecut2 knockout mice, their function as a critical component in the process of horizontal interneuron differentiation was determined. Second, computational analyses determined that among the genes most highly correlated with Math5 expression in the same dataset of retinal progenitors was Polo-like kinase 3 (Plk3). After preliminary exploration of the expression patterns of Plk3 determined that it was present in retinal progenitors at an optimal time and location to affect retinal development, a Plk3 knockout mouse was also characterized to determine its function in retinogenesis. Lastly, single cells were isolated from the chicken as a

starting point in a comparative transcriptomics approach to determine evolutionarily conserved patterns in gene expression among vertebrate species. Taken all together, the studies I have undertaken have shown that approaching complex developmental processes such as retinogenesis using a single-cell resolution may provide an unprecedented understanding of the processes that drive the differentiation of not only large categories of retinal cells, but also distinctive subsets.

7. References

Adler, R., and Canto-Soler, M.V. (2007). Molecular mechanisms of optic vesicle development: Complexities, ambiguities and controversies. Dev. Biol. *305*, 1–13.

Applebury, M.., Antoch, M.., Baxter, L.., Chun, L.L.., Falk, J.., Farhangfar, F., Kage, K., Krzystolik, M.., Lyass, L.., and Robbins, J.. (2000). The Murine Cone PhotoreceptorA Single Cone Type Expresses Both S and M Opsins with Retinal Spatial Patterning. Neuron *27*, 513–523.

Baas, D., Bumsted, K.M., Martinez, J.A., Vaccarino, F.M., Wikler, K.C., and Barnstable, C.J. (2000). The subcellular localization of Otx2 is cell-type specific and developmentally regulated in the mouse retina. Brain Res. Mol. Brain Res. *78*, 26–37.

Baye, L.M., and Link, B.A. (2007). Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis. J Neurosci *27*, 10143–10152.

Belliveau, M.J., and Cepko, C.L. (1999). Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. Development *126*, 555–566.

Belliveau, M.J., Young, T.L., and Cepko, C.L. (2000). Late retinal progenitor cells show intrinsic limitations in the production of cell types and the kinetics of opsin synthesis. J Neurosci *20*, 2247–2254.

Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W.P., Weber, G., Lee, K., Fraioli, R.E., Cho, S.-H., et al. (2004). Genomic Analysis of Mouse Retinal Development. PLoS Biol. *2*, e247.

Brown, N.L., Kanekar, S., Vetter, M.L., Tucker, P.K., Gemza, D.L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Dev. Camb. Engl. *125*, 4821–4833.

Brown, N.L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Dev. Camb. Engl. *128*, 2497–2508.

Bruhn, S.L., and Cepko, C.L. (1996). Development of the pattern of photoreceptors in the chick retina. J. Neurosci. Off. J. Soc. Neurosci. *16*, 1430–1439.

Brzezinski, J.A. th, Kim, E.J., Johnson, J.E., and Reh, T.A. (2011). Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development *138*, 3519–3531.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., et al. (1996). Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat Genet *12*, 376–384.

Burns, M.E., and Arshavsky, V.Y. (2005). Beyond counting photons: trials and trends in vertebrate visual transduction. Neuron *48*, 387–401.

Cayouette, M., and Raff, M. (2003). The orientation of cell division influences cell-fate choice in the developing mammalian retina. Development *130*, 2329–2339.

Cayouette, M., Barres, B.A., and Raff, M. (2003). Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron *40*, 897–904.

Cepko, C.L. (1996). The patterning and onset of opsin expression in vertebrate retinae. Curr. Opin. Neurobiol. *6*, 542–546.

Cepko, C.L., Austin, C.P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U A *93*, 589–595.

Chen, S., Wang, Q.L., Nie, Z., Sun, H., Lennon, G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Zack, D.J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron *19*, 1017–1030.

Cherry, T.J., Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2009). Development and diversification of retinal amacrine interneurons at single cell resolution. Proc. Natl. Acad. Sci. *106*, 9495–9500.

Cherry, T.J., Wang, S., Bormuth, I., Schwab, M., Olson, J., and Cepko, C.L. (2011). NeuroD factors regulate cell fate and neurite stratification in the developing retina. J Neurosci *31*, 7365–7379.

Cheung, D.W.S., Wai, M.S.M., and Yew, D.T. (2013). The diversity of cones in the retina of vertebrates: a review. OA Anat. *1*, 4.

Chowers, I., Gunatilaka, T.L., Farkas, R.H., Qian, J., Hackam, A.S., Duh, E., Kageyama, M., Wang, C., Vora, A., Campochiaro, P.A., et al. (2003). Identification of novel genes preferentially expressed in the retina using a custom human retina cDNA microarray. Invest Ophthalmol Vis Sci *44*, 3732–3741.

Dräger, U.C., and Olsen, J.F. (1981). Ganglion cell distribution in the retina of the mouse. Invest. Ophthalmol. Vis. Sci. *20*, 285–293.

Dyer, M.A. (2003). Regulation of proliferation, cell fate specification and differentiation by the homeodomain proteins Prox1, Six3, and Chx10 in the developing retina. Cell Cycle *2*, 350–357.

Dyer, M.A., and Cepko, C.L. (2000). p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Dev. Camb. Engl. *127*, 3593–3605.

Dyer, M.A., and Cepko, C.L. (2001a). Regulating proliferation during retinal development. Nat Rev Neurosci *2*, 333–342.

Dyer, M.A., and Cepko, C.L. (2001b). p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations. J Neurosci *21*, 4259–4271.

Elliott, J., Jolicoeur, C., Ramamurthy, V., and Cayouette, M. (2008). Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron *60*, 26–39.

Enright, J.M., Lawrence, K.A., Hadzic, T., and Corbo, J.C. (2015). Transcriptome profiling of developing photoreceptor subtypes reveals candidate genes involved in avian photoreceptor diversification: Transcriptome Profiling of Avian Photoreceptors. J. Comp. Neurol. *523*, 649–668.

Farah, M.H., and Easter, S.S. (2005). Cell birth and death in the mouse retinal ganglion cell layer. J. Comp. Neurol. *489*, 120–134.

Fausett, B.V., Gumerson, J.D., and Goldman, D. (2008). The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci *28*, 1109–1117.

Feng, L., Xie, X., Joshi, P.S., Yang, Z., Shibasaki, K., Chow, R.L., and Gan, L. (2006). Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. Development *133*, 4815–4825.

Feng, L., Xie, Z., Ding, Q., Xie, X., Libby, R.T., and Gan, L. (2010). MATH5 controls the acquisition of multiple retinal cell fates. Mol. Brain *3*, 36.

Field, G.D., and Chichilnisky, E.J. (2007). Information Processing in the Primate Retina: Circuitry and Coding. Annu. Rev. Neurosci. *30*, 1–30.

Fischer, A.J., Stanke, J.J., Aloisio, G., Hoy, H., and Stell, W.K. (2007). Heterogeneity of horizontal cells in the chicken retina. J. Comp. Neurol. *500*, 1154–1171.

Fuhrmann, S. (2010). Eye Morphogenesis and Patterning of the Optic Vesicle. In Current Topics in Developmental Biology, (Elsevier), pp. 61–84.

Furukawa, T., Morrow, E.M., and Cepko, C.L. (1997). Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell *91*, 531–541.

Furukawa, T., Mukherjee, S., Bao, Z.Z., Morrow, E.M., and Cepko, C.L. (2000). rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells. Neuron *26*, 383–394.

Gamsiz, E.D., Ouyang, Q., Schmidt, M., Nagpal, S., and Morrow, E.M. (2012). Genome-wide transcriptome analysis in murine neural retina using high-throughput RNA sequencing. Genomics *99*, 44–51.

Gan, L., Xiang, M., Zhou, L., Wagner, D.S., Klein, W.H., and Nathans, J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. Proc Natl Acad Sci U A *93*, 3920–3925.

Goetz, J.J., and Trimarchi, J.M. (2012). Single-cell profiling of developing and mature retinal neurons. J Vis Exp.

Gomes, F.L., Zhang, G., Carbonell, F., Correa, J.A., Harris, W.A., Simons, B.D., and Cayouette, M. (2011). Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. Development *138*, 227–235.

Green, E.S., Stubbs, J.L., and Levine, E.M. (2003). Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development *130*, 539–552.

Grimes, W.N., Schwartz, G.W., and Rieke, F. (2014). The Synaptic and Circuit Mechanisms Underlying a Change in Spatial Encoding in the Retina. Neuron *82*, 460–473.

Grindley, J.C., Davidson, D.R., and Hill, R.E. (1995). The role of Pax-6 in eye and nasal development. Development *121*, 1433–1442.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., and Joyner, A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463–476.

Hafler, B.P., Surzenko, N., Beier, K.T., Punzo, C., Trimarchi, J.M., Kong, J.H., and Cepko, C.L. (2012). Transcription factor Olig2 defines subpopulations of retinal progenitor cells biased toward specific cell fates. Proc Natl Acad Sci U A *109*, 7882–7887.

Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. Science *267*, 1788–1792.

Hatakeyama, J., and Kageyama, R. (2004). Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol *15*, 83–89.

Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. Development *128*, 1313–1322.

He, J., Zhang, G., Almeida, A.D., Cayouette, M., Simons, B.D., and Harris, W.A. (2012). How Variable Clones Build an Invariant Retina. Neuron *75*, 786–798.

Hobert, O. (2010). Neurogenesis in the nematode Caenorhabditis elegans. WormBook 1–24.

Holt, C.E., Bertsch, T.W., Ellis, H.M., and Harris, W.A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron *1*, 15–26.

Hu, M., and Easter, S.S. (1999). Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. Dev. Biol. *207*, 309–321.

De la Huerta, I., Kim, I.-J., Voinescu, P.E., and Sanes, J.R. (2012). Direction-selective retinal ganglion cells arise from molecularly specified multipotential progenitors. Proc. Natl. Acad. Sci. *109*, 17663–17668.

Hufnagel, R.B., Riesenberg, A.N., Quinn, M., Brzezinski, J.A. th, Glaser, T., and Brown, N.L. (2013). Heterochronic misexpression of Ascl1 in the Atoh7 retinal cell lineage blocks cell cycle exit. Mol Cell Neurosci *54*, 108–120.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J.E., and Kageyama, R. (2002). Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development *129*, 831–842.

Isshiki, T., Pearson, B., Holbrook, S., and Doe, C.Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell *106*, 511–521.

Jarman, A.P., Grell, E.H., Ackerman, L., Jan, L.Y., and Jan, Y.N. (1994). Atonal is the proneural gene for Drosophila photoreceptors. Nature *369*, 398–400.

Jarman, A.P., Sun, Y., Jan, L.Y., and Jan, Y.N. (1995). Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. Development *121*, 2019–2030.

Johnston, R.J., and Desplan, C. (2008). Stochastic neuronal cell fate choices. Curr Opin Neurobiol *18*, 20–27.

Kanekar, S., Perron, M., Dorsky, R., Harris, W.A., Jan, L.Y., Jan, Y.N., and Vetter, M.L. (1997). Xath5 Participates in a Network of bHLH Genes in the Developing Xenopus Retina. Neuron *19*, 981–994.

Karl, M.O., Hayes, S., Nelson, B.R., Tan, K., Buckingham, B., and Reh, T.A. (2008). Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U A *105*, 19508–19513.

Kay, J.N., Finger-Baier, K.C., Roeser, T., Staub, W., and Baier, H. (2001). Retinal Ganglion Cell Genesis Requires lakritz, a Zebrafish atonal Homolog. Neuron *30*, 725–736.

Kay, J.N., De la Huerta, I., Kim, I.J., Zhang, Y., Yamagata, M., Chu, M.W., Meister, M., and Sanes, J.R. (2011). Retinal ganglion cells with distinct directional preferences differ in molecular identity, structure, and central projections. J Neurosci *31*, 7753–7762.

Kechad, A., Jolicoeur, C., Tufford, A., Mattar, P., Chow, R.W., Harris, W.A., and Cayouette, M. (2012). Numb is required for the production of terminal asymmetric cell divisions in the developing mouse retina. J Neurosci *32*, 17197–17210.

Kim, D.S., Ross, S.E., Trimarchi, J.M., Aach, J., Greenberg, M.E., and Cepko, C.L. (2008). Identification of molecular markers of bipolar cells in the murine retina. J. Comp. Neurol. *507*, 1795–1810.

Koike, C., Nishida, A., Ueno, S., Saito, H., Sanuki, R., Sato, S., Furukawa, A., Aizawa, S., Matsuo, I., Suzuki, N., et al. (2007). Functional roles of Otx2 transcription factor in postnatal mouse retinal development. Mol. Cell. Biol. *27*, 8318–8329.

Kuehn, M.H., Fingert, J.H., and Kwon, Y.H. (2005). Retinal ganglion cell death in glaucoma: mechanisms and neuroprotective strategies. Ophthal Clin North Am *18*, 383–395.

Lee, J.E. (1997). Basic helix-loop-helix genes in neural development. Curr Opin Neurobiol *7*, 13–20.

Li, S., Mo, Z., Yang, X., Price, S.M., Shen, M.M., and Xiang, M. (2004). Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron *43*, 795–807.

Liu, W., Mo, Z., and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. Proc. Natl. Acad. Sci. U. S. A. *98*, 1649–1654.

Livesey, F.J., and Cepko, C.L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. *2*, 109–118.

Livesey, F.J., Young, T.L., and Cepko, C.L. (2004). An analysis of the gene expression program of mammalian neural progenitor cells. Proc Natl Acad Sci U A *101*, 1374–1379.

Luo, H., Jin, K., Xie, Z., Qiu, F., Li, S., Zou, M., Cai, L., Hozumi, K., Shima, D.T., and Xiang, M. (2012). Forkhead box N4 (Foxn4) activates Dll4-Notch signaling to suppress photoreceptor cell fates of early retinal progenitors. Proc Natl Acad Sci U A *109*, E553–E562.

MacNeil, M.A., and Masland, R.H. (1998). Extreme diversity among amacrine cells: implications for function. Neuron *20*, 971–982.

Mao, C.A., Wang, S.W., Pan, P., and Klein, W.H. (2008). Rewiring the retinal ganglion cell gene regulatory network: Neurod1 promotes retinal ganglion cell fate in the absence of Math5. Development *135*, 3379–3388.

Mao, C.A., Cho, J.H., Wang, J., Gao, Z., Pan, P., Tsai, W.W., Frishman, L.J., and Klein, W.H. (2013). Reprogramming amacrine and photoreceptor progenitors into retinal ganglion cells by replacing Neurod1 with Atoh7. Development *140*, 541–551.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., and Gruss, P. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. Cell *105*, 43–55.

Martinez-Morales, J.R., and Wittbrodt, J. (2009). Shaping the vertebrate eye. Curr. Opin. Genet. Dev. *19*, 511–517.

Masland, R. (1988). Amacrine cells. Trends Neurosci. *11*, 405–410.

Masland, R.H. (2001a). The fundamental plan of the retina. Nat. Neurosci. *4*, 877–886.

Masland, R.H. (2001b). Neuronal diversity in the retina. Curr Opin Neurobiol *11*, 431–436.

Masland, R.H. (2012). The neuronal organization of the retina. Neuron *76*, 266–280.

Masland, R.H., and Raviola, E. (2000). Confronting complexity: strategies for understanding the microcircuitry of the retina. Annu Rev Neurosci *23*, 249–284.

Mathers, P.H., Grinberg, A., Mahon, K.A., and Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. Nature *387*, 603–607.

Matsuda, T., and Cepko, C.L. (2007). Controlled expression of transgenes introduced by in vivo electroporation. Proc Natl Acad Sci U A *104*, 1027–1032.

Mattar, P., Ericson, J., Blackshaw, S., and Cayouette, M. (2015). A Conserved Regulatory Logic Controls Temporal Identity in Mouse Neural Progenitors. Neuron *85*, 497–504.

Miller, A.C., Seymour, H., King, C., and Herman, T.G. (2008). Loss of seven-up from Drosophila R1/R6 photoreceptors reveals a stochastic fate choice that is normally biased by Notch. Development *135*, 707–715.

Morrow, E.M., Furukawa, T., and Cepko, C.L. (1998). Vertebrate photoreceptor cell development and disease. Trends Cell Biol. *8*, 353–358.

Morrow, E.M., Furukawa, T., Lee, J.E., and Cepko, C.L. (1999). NeuroD regulates multiple functions in the developing neural retina in rodent. Development *126*, 23–36.

Mu, X., Zhao, S., Pershad, R., Hsieh, T.F., Scarpa, A., Wang, S.W., White, R.A., Beremand, P.D., Thomas, T.L., Gan, L., et al. (2001). Gene expression in the developing mouse retina by EST sequencing and microarray analysis. Nucleic Acids Res *29*, 4983–4993.

Mu, X., Fu, X., Sun, H., Beremand, P.D., Thomas, T.L., and Klein, W.H. (2005). A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. Dev Biol *280*, 467–481.

Muranishi, Y., Terada, K., Inoue, T., Katoh, K., Tsujii, T., Sanuki, R., Kurokawa, D., Aizawa, S., Tamaki, Y., and Furukawa, T. (2011). An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. J. Neurosci. Off. J. Soc. Neurosci. *31*, 16792–16807.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., et al. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537–544.

Naito, J., and Chen, Y. (2004). Morphological features of chick retinal ganglion cells. Anat. Sci. Int. *79*, 213–225.

Nelson, S.M., Park, L., and Stenkamp, D.L. (2009). Retinal homeobox 1 is required for retinal neurogenesis and photoreceptor differentiation in embryonic zebrafish. Dev Biol *328*, 24–39.

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., and Furukawa, T. (2003). Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci *6*, 1255–1263.

Nornes, S., Clarkson, M., Mikkola, I., Pedersen, M., Bardsley, A., Martinez, J.P., Krauss, S., and Johansen, T. (1998). Zebrafish contains two pax6 genes involved in eye development. Mech Dev *77*, 185–196.

Ochocinska, M.J., and Hitchcock, P.F. (2009). NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. Mech Dev *126*, 128–141.

Ohnuma, S., Philpott, A., Wang, K., Holt, C.E., and Harris, W.A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. Cell *99*, 499–510.

Oron-Karni, V., Farhy, C., Elgart, M., Marquardt, T., Remizova, L., Yaron, O., Xie, Q., Cvekl, A., and Ashery-Padan, R. (2008). Dual requirement for Pax6 in retinal progenitor cells. Development *135*, 4037–4047.

Pearson, B.J., and Doe, C.Q. (2004). Specification of temporal identity in the developing nervous system. Annu Rev Cell Dev Biol *20*, 619–647.

Peichl, L., and González-Soriano, J. (1994). Morphological types of horizontal cell in rodent retinae: a comparison of rat, mouse, gerbil, and guinea pig. Vis. Neurosci. *11*, 501–517.

Pollak, J., Wilken, M.S., Ueki, Y., Cox, K.E., Sullivan, J.M., Taylor, R.J., Levine, E.M., and Reh, T.A. (2013). ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development *140*, 2619–2631.

Prasov, L., and Glaser, T. (2012). Pushing the envelope of retinal ganglion cell genesis: context dependent function of Math5 (Atoh7). Dev Biol *368*, 214–230.

Riesenberg, A.N., Le, T.T., Willardsen, M.I., Blackburn, D.C., Vetter, M.L., and Brown, N.L. (2009). Pax6 regulation of Math5 during mouse retinal neurogenesis. Genesis *47*, 175–187.

Rockhill, R.L., Daly, F.J., MacNeil, M.A., Brown, S.P., and Masland, R.H. (2002). The diversity of ganglion cells in a mammalian retina. J. Neurosci. Off. J. Soc. Neurosci. *22*, 3831–3843.

Rodieck, R.W. (1998). The first steps in seeing (Sunderland, Mass: Sinauer Associates).

Roegiers, F., and Jan, Y.N. (2004). Asymmetric cell division. Curr Opin Cell Biol *16*, 195–205.

Schmeer, C.W., Wohl, S.G., and Isenmann, S. (2012). Cell-replacement therapy and neural repair in the retina. Cell Tissue Res *349*, 363–374.

Sidman, R. (1961). Histogenesis of mouse retina studied with thymidine-H3. In The Structure of the Eye, G. Smelser, ed. (New York: Academic Press), pp. 487–506.

Smirnova, L., Gräfe, A., Seiler, A., Schumacher, S., Nitsch, R., and Wulczyn, F.G. (2005). Regulation of miRNA expression during neural cell specification. Eur. J. Neurosci. *21*, 1469–1477.

Snow, R.L., and Robson, J.A. (1994). Ganglion cell neurogenesis, migration and early differentiation in the chick retina. Neuroscience *58*, 399–409.

Spence, S.G., and Robson, J.A. (1989). An autoradiographic analysis of neurogenesis in the chick retina in vitro and in vivo. Neuroscience *32*, 801–812.

Strettoi, E., Raviola, E., and Dacheux, R.F. (1992). Synaptic connections of the narrow-field, bistratified rod amacrine cell (AII) in the rabbit retina. J. Comp. Neurol. *325*, 152–168.

Swaroop, A., and Zack, D.J. (2002). Transcriptome analysis of the retina. Genome Biol *3*, REVIEWS1022.

Thoreson, W.B., and Mangel, S.C. (2012). Lateral interactions in the outer retina. Prog. Retin. Eye Res. *31*, 407–441.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996). Mammalian hairy and Enhancer of split homolog 1

regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron *16*, 723–734.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J *19*, 5460–5472.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual Retinal Progenitor Cells Display Extensive Heterogeneity of Gene Expression. PLoS ONE *3*, e1588.

Tucker, B.A., Park, I.H., Qi, S.D., Klassen, H.J., Jiang, C., Yao, J., Redenti, S., Daley, G.Q., and Young, M.J. (2011). Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. PLoS ONE *6*, e18992.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Vaney, D.I., Peichl, L., and Boycott, B.B. (1988). Neurofibrillar long-range amacrine cells in mammalian retinae. Proc R Soc Lond B Biol Sci *235*, 203–219.

Voinescu, P.E., Kay, J.N., and Sanes, J.R. (2009). Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. J. Comp. Neurol. *517*, 737–750.

Wang, S.W. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev. *15*, 24–29.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. *10*, 57–63.

Wässle, H. (2004). Parallel processing in the mammalian retina. Nat. Rev. Neurosci. *5*, 747–757.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Yang, Z., Ding, K., Pan, L., Deng, M., and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. *264*, 240–254.

Yau, K.W. (1994). Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. Invest. Ophthalmol. Vis. Sci. *35*, 9–32.

Young, R.W. (1985). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

Zhang, J., Gray, J., Wu, L., Leone, G., Rowan, S., Cepko, C.L., Zhu, X., Craft, C.M., and Dyer, M.A. (2004). Rb regulates proliferation and rod photoreceptor development in the mouse retina. Nat Genet *36*, 351–360.

8. Figures and Legends

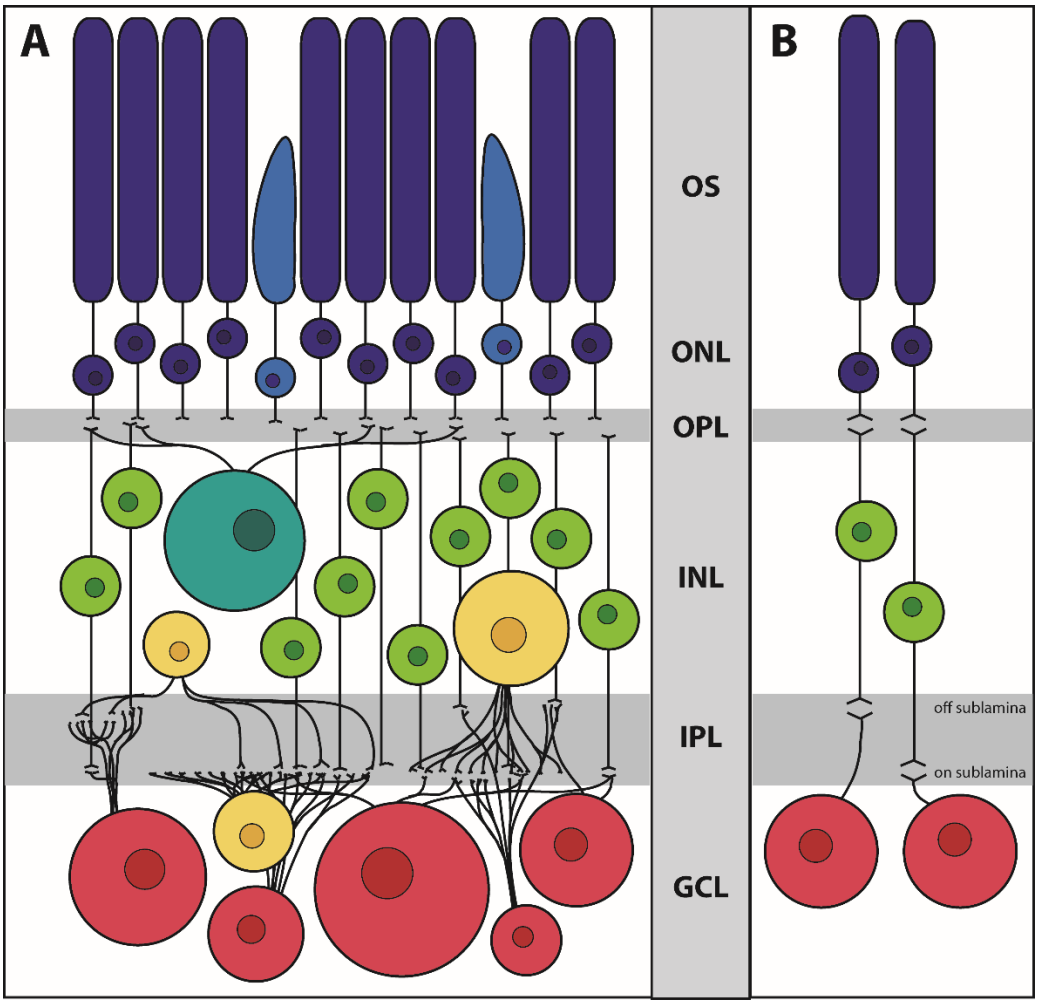
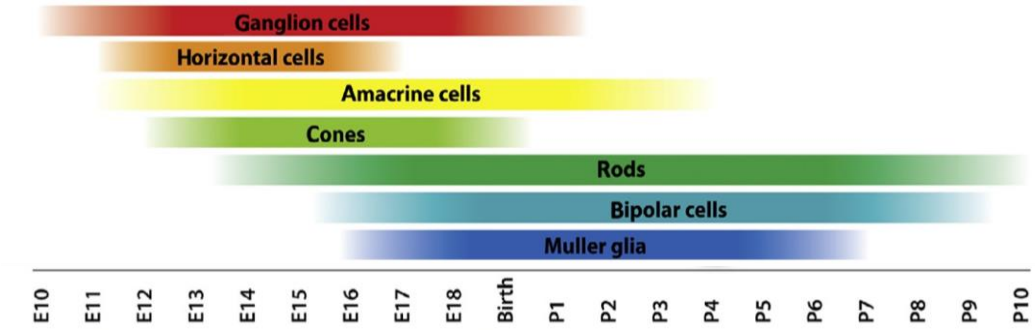


Figure 1. Retinal organization.

(A) The organization of the vertebrate retina. Retinal layers are labeled, including the photoreceptor outer segments (OS), the outer nuclear layer (ONL) containing photoreceptor cell bodies, the outer plexiform layer (OPL) containing synapses between photoreceptors and interneurons, the cell bodies of which are located in the inner nuclear layer (INL). The inner plexiform layer (IPL) separates the INL from the ganglion cell layer (GCL) containing both ganglion cells and displaced amacrine interneurons. Some cell types, such as the red ganglion cells or yellow amacrine cells, show diverse morphologies in both size and stratification of connectivity. (B) Examples of the diversity within the major kinds of retinal cells. Bipolar cells are largely classified as either ON or OFF based on the location and connectivity of their dendrites within the sublaminae of the IPL, which correspond to their reactivity to light.

A



B

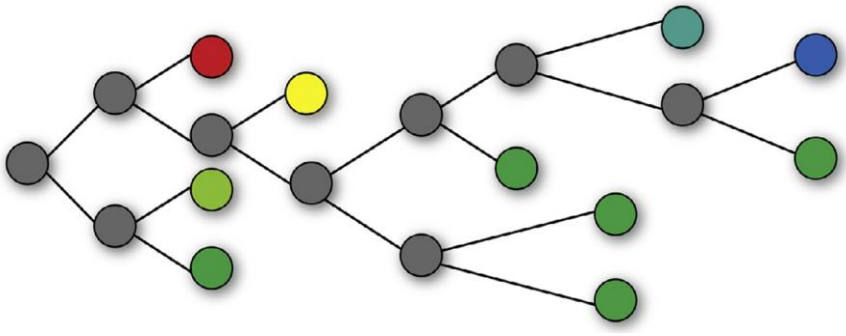


Figure 2. The competence model of retinal development.

(A) A timeline of retinal progenitor cell competence during mouse development is shown at the top. This diagram demonstrates the distinct but overlapping timepoints during which a progenitor cell may generate a given type of retinal neuron. (B) The lineage that is displayed is from a hypothetical retinal progenitor cell. Although the ratios of cells generated from any given progenitor cell are not directly proportionate to the retina as a whole, this figure illustrates progenitor multipotency. A single progenitor cell may undergo a proliferative division yielding two progenitor cells for daughters, a neurogenic division resulting in two differentiated neurons, or an asymmetric division to produce a differentiated neuron as well as a dividing progenitor. It is important to note that while progenitor cells appear to be capable of generating any given retinal cell type, a single progenitor does not necessarily produce them all.

CHAPTER 2. ONECUT1 AND ONECUT2 PLAY CRITICAL ROLES IN THE DEVELOPMENT OF THE MOUSE RETINA

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1. Abstract

The entire repertoire of intrinsic factors that control the cell fate determination process of specific retinal neurons has yet to be fully identified. Single cell transcriptome profiling experiments of retinal progenitor cells revealed considerable gene expression heterogeneity between individual cells, especially among different classes of transcription factors. In this study, we show that two of those factors, Onecut1 and Onecut2, are expressed during mouse retinal development. Using mice that are deficient for each of these transcription factors, we further demonstrate a significant loss (~70-80%) of horizontal cells in the absence of either of these proteins, while the other retinal cells appear at normal numbers. Microarray profiling experiments performed on knockout retinas revealed defects in horizontal cell genes as early as E14.5. Additional profiling assays showed an upregulation of several stress response genes in the adult Onecut2 knockout, suggesting that the integrity of the retina is compromised in the absence of normal numbers of horizontal cells. Interestingly, melanopsin, the gene coding for the photopigment found in photosensitive ganglion cells, was observed to be upregulated in Onecut1 deficient retinas, pointing to a possible regulatory role for Onecut1. Taken together, our data show that similar to Onecut1, Onecut2 is also necessary for the formation of normal numbers of horizontal cells in the developing retina.

2. Introduction

Cell fate determination is an intricate process that is controlled by combinations of genes, which have not yet been fully identified. A better understanding of how cellular diversity arises in vertebrates can help us comprehend precisely how cells become specialized to perform specific functions within a complex tissue. Diversity is an especially critical component of the central nervous system's ability to perform complex tasks such as sensory processing. Developing neural progenitors must integrate extrinsic signals from their environment and neighboring cells, as well as intrinsic cues (usually in the form of transcription factors), in order to make appropriate cell fate determinations. The manner in which the progenitor cell in question interprets these signals varies in different tissues (Edlund and Jessell, 1999; Goetz et al., 2014). In a deterministic model of cell fate acquisition, these signals act to facilitate the generation of specific repertoires of daughter cells in a stereotyped fashion (He et al., 2012; Livesey and Cepko, 2001). Conversely, a stochastic or probabilistic model of cell-fate determination proposes a more fluid development, wherein the dynamic nature of intrinsic and extrinsic signals lead to changing probabilities of a progenitor cell generating various different cell types (Chen et al., 2012; He et al., 2012). Although evidence for theories of neurogenesis exists in different model systems (Chen et al., 2012; Isshiki et al., 2001), recent studies in zebrafish suggest that stochastic probabilities play a role in the cell fate determination of the developing retina (He et al., 2012).

The developing retina is an excellent model to study neural cell fate determination due to its relatively simple organization and ease of accessibility. During retinogenesis, a combination of intrinsic and extrinsic signals drives a common pool of retinal progenitor cells to generate a functioning tissue with the correct proportions of six different neurons and one glial cell type (Livesey and Cepko, 2001; Turner and Cepko, 1987; Turner et al., 1990). The mature retina is organized into three cell layers: an outer nuclear layer (ONL) consisting of the two types of photoreceptors, rods and cones; an inner nuclear layer (INL) containing horizontal, bipolar, and amacrine interneurons; and lastly, a retinal ganglion cell layer (GCL) composed of displaced amacrine cells and ganglion cells, whose long axons comprise the optic nerve and communicate visual signals to the cortex (Masland, 2001). During development each retinal cell type is generated at overlapping yet distinctive timepoints from a common pool of progenitor cells (Turner and Cepko, 1987). This timeline of generation is stereotypical among vertebrates, with ganglion cells generated first, followed closely by early-born amacrine cells, horizontal cells, and cone photoreceptors (Cherry et al., 2009; Sidman, 1961; Voinescu et al., 2009; Young, 1985a, 1985b). These early-generated cells are followed by the production of the later-born bipolar cells and the sole retinal glia type, the Muller glia, while the large population of rod photoreceptors is generated throughout retinal development (Cepko, 1996; Sidman, 1961; Young, 1985a, 1985b). Identifying the factors that drive retinal progenitor cells to one cell fate versus another can be challenging, especially when attempting to focus on those that drive the generation of rare yet functionally critical neurons, such as ganglion

cells or horizontal cells. Additionally, even retinal progenitor cells that will eventually produce the same type of neuron may be at various stages of development at any given point during retinogenesis. Whole-tissue approaches aimed at uncovering the transcriptomes of progenitor cells during cell fate determination can drown out the signals of rare cells or dynamic changes within certain progenitor cells at various stages of development. Therefore, a single-cell approach was previously utilized to profile the transcriptomic signatures of individual progenitor cells from multiple stages of mouse development (Trimarchi et al., 2008). These single cell transcriptomes revealed considerable gene expression heterogeneity among retinal progenitor cells, especially when transcription factors were specifically examined.

One intrinsic factor that is important for retinal development, Math5, is a basic helix-loop-helix (bHLH) transcription factor that is expressed in subsets of retinal progenitor cells in the embryonic retina (Brzezinski et al., 2012; Skowronska-Krawczyk et al., 2009). Lineage tracing studies have shown Math5 expression in progenitor cells that generated early-born retinal cell types, including rod and cone photoreceptors, amacrine cells, horizontal cells, and ganglion cells (Feng et al., 2010; Yang et al., 2003). Loss of Math5 leads to an 80-90% decrease in retinal ganglion cells in mice and a complete loss of ganglion cells in the zebrafish (Brown et al., 2001; Kay et al., 2001; Wang, 2001). Concomitantly, the absence of this transcription factor led to an increase in the number of cone photoreceptors and subsets of amacrine cells (Brown et al., 2001; Wang, 2001), indicating that it plays a critical role in the normal development of multiple cell types. Given the

expression of Math5 and its importance in retinal development, the transcriptomes of single cells expressing Math5 were examined to identify genes involved in the acquisition of early retinal cell fates. These single cell profiles were derived from both progenitor cells isolated at random (Trimarchi et al., 2008) and targeted cells isolated from Math5-LacZ mice (Trimarchi et al., in preparation).

Transcription factors present in subsets of Math5-expressing (Math5^{+}) cells were of particular interest, as they were hypothesized to play a role in the activation of downstream transcriptomic programs that lead to the determination of particular retinal cell fates. Two such factors, Onecut1 and Onecut2, were identified through their expression in subsets of Math5^{+} cells. Additionally, these factors have recently been shown in a separate study to be expressed during retinal development (Wu et al., 2012). The Onecut transcription factor family contains three members named for their characteristic structure that includes a single cut domain and a homeodomain (Clotman et al., 2002; Lemaigre et al., 1996). Previously, the Onecut transcription factors have been demonstrated to play critical roles in the development of the liver, bile duct, pancreas and the immune system (Clotman et al., 2002; Furuno et al., 2008; Jacquemin et al., 2003). In this study, two members of the Onecut family, Onecut1 (OC1) and Onecut2 (OC2), were found to be correlated with Math5 expression in the transcriptomes of individual retinal progenitors. The role of Onecut1 (OC1) in retinal cell fate has recently been investigated (Wu et al., 2013), but the functionality of its family member Onecut2 (OC2) in the developing retina has not been explored. In this study we examined the retinal phenotypes resulting from the loss of either OC1 or OC2 in mice. In mice deficient for either factor, we

observed a significant decrease in horizontal cells, while the other retinal cell populations remained grossly the same. Through the use of microarray profiling, we further characterized both OC1 and OC2 deficient mice. Our results suggest that these transcription factors have significant redundancy in their regulation of horizontal cell genes and reveal possible differences in the regulation of genes in other retinal cell lineages.

3. Materials and methods

Ethics statement

All procedures for the care and housing of mice conform to the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Tissue preparation

Whole eyes were removed and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) at 4°C for 1 hour. Following fixation, eyes were rinsed 3 times in PBS at 4°C for 10 minutes. The sclera was dissected away from the intact lens and retina. Retinas (with lens intact) were post-fixed in 4% paraformaldehyde/PBS at

4°C for 15 minutes and then rinsed 3 times with PBS. The lens was removed and the retina taken for further processing depending upon the particular assay.

Whole-mount immunohistochemistry

Dissected retinas were equilibrated at 4°C in sucrose solutions of increasing concentrations in a step-wise manner, 10%, 20%, 30% sucrose (w/v) in PBS, for at least 30 minutes or until the retina had settled to the bottom of the tube. While the retinas were in 30% sucrose/PBS, they were snap-frozen on dry ice and subjected to three freeze/thaw cycles. Retinas were stored at -80°C in the 30% sucrose/PBS solution or used for immunostaining immediately. Following freeze/thaw, retinas were rinsed 3 times in PBS for 30 minutes and blocked for 2 hours at room temperature (RT) in blocking solution [3% goat serum/1% bovine serum albumin (BSA)/0.1% Triton-X100/0.02% sodium dodecyl sulfate (SDS) in PBS]. Retinas were then incubated in primary antibody in blocking solution overnight at 4°C on a rocking platform. The next day, retinas were rinsed 3 times in PBS for 30 minutes and placed in secondary antibody in blocking solution overnight at 4°C on a rocking platform. Retinas were rinsed 3 times in PBS for 30 minutes and then flattened between two coverslips for confocal imaging on a Leica SP5 X MP confocal microscope.

Tissue sectioning

Dissected retinas were equilibrated in 30% sucrose/PBS at 4°C until they sank to the bottom of the tube. They were then frozen in an equilibrated solution of 50% (30% sucrose/PBS): 50% optimal cutting temperature compound (OCT, Tissue-Tek; Sakura Finetek, Torrance, CA) and stored at -80°C. Cryosections were cut at 20 μm, collected on Superfrost Plus slides (Fisher Scientific), and air-dried for 30 minutes at RT before storage at -80°C.

Section antibody staining

Sections were incubated in blocking solution (1% BSA/0.1 Triton-X100 in PBS) for 1 hour at RT and then in primary antibody in blocking solution overnight at 4°C. Slides were rinsed in blocking solution 3 times and incubated with secondary antibody in blocking solution for 2 hours at RT. Slides were rinsed with PBS, mounted in Fluoromount-G and visualized on a Leica SP5 X MP confocal microscope.

Primary antibodies used were anti-Calbindin28K (1:2000) (Swant, Switzerland), anti-Chx10 (1:1000) (Morrow et al., 2008), anti-Glutamine Synthetase (1:10,000) (Sigma), anti-Rhodopsin (1:100) (Molday and MacKenzie, 1983), anti-GFAP (1:100) (Abcam), and anti-HNF-6/OC1 (1:200) (Santa Cruz Biotechnology). The anti-Pax6 (1:50) and anti-Islet1 (1:50) antibodies were obtained from the Developmental Studies Hybridoma Bank (DHSB), developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City.

Quantitative immunohistochemical analysis

For analysis, identically-sized fields of immunostained retinal cryosections were counted by hand in Adobe Photoshop, blinded to genotype. After counts were made for WT and KO fields (n=3 biological replicates for both - except in the case of Calb28k, in which 4 fields of both amacrine cell staining and horizontal cell staining were counted), t-tests were performed to compare counts of the WT and KO fields. Error bars indicate standard error.

Section *in situ* hybridization

Riboprobe synthesis – Probe template sequences were amplified from mouse cDNA, cloned into the pGEM-T vector (Promega) and sequenced. Antisense riboprobes were transcribed using either T7 or Sp6 RNA polymerase in the presence of digoxigenin (DIG) or fluorescein-labeled nucleotides for 1-2 hours at 37°C. Riboprobes were treated with DNase I for 15 minutes at 37°C and precipitated overnight with LiCl and 100% ethanol.

In Situ *Hybridizations (ISH)* – *In situ* hybridizations on retinal cryosections and dissociated cells were performed exactly as described in Trimarchi et al. 2007. For the dissociated ISH, one probe was synthesized with a digoxigenin (DIG)-label and the other probe was labeled with fluorescein. DIG-labeled probes were detected using an anti-DIG-POD antibody (Roche, 1:1000) and a Cy3 tyramide solution (PerkinElmer), while fluorescein-labeled probes were detected using an anti-fluorescein-POD antibody (Roche, 1:1000) and an Alexa-488 tyramide (Life

Technologies). Tyramide amplification (Life Technologies) was performed for 10 minutes for the DIG-labeled probe, followed by inactivation in 0.3% hydrogen peroxide and tyramide amplification for the fluorescein-labeled probe. The slides were fixed in 4% paraformaldehyde and mounted. Six independent fields were photographed and quantified for each.

Microarray experiments

Total RNA Isolation – RNA was extracted from retinal tissue using TRI-Reagent (Sigma) according to the manufacturer's instructions. Briefly, tissue was homogenized in 1 ml of TRI-Reagent, 0.1 ml of 1-bromo-3-chloropropane was added to sample, followed by vigorous shaking for 15 seconds. Samples were allowed to sit at RT for 10 minutes and centrifuged at 13,300 rpm for 15 minutes at 4°C, and the resulting aqueous phase was isolated. RNA was precipitated by adding 0.5 ml of isopropanol per 1 ml of TRI-Reagent and allowing the sample to incubate at RT for 10 minutes followed by centrifugation at 13,300 rpm for 10 minutes at 4°C.

RNA Amplification – Total RNA from retinal tissue was amplified, biotinylated, and fragmented using the MessageAmp III RNA Amplification Kit (Ambion), following the manufacturer's instructions. All steps were carried out in a thermal cycler. 10 μg of amplified RNA was fragmented for all of the directly compared samples. Amplified RNA was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays at the Iowa State University GeneChip Facility. Microarray analysis was performed using the Affy R package developed by Bioconductor. After

background adjustment and normalization using Mas5, the data were log(2) transformed. To be considered for differential expression analysis, the log(2) transformed mean of either n=3 WT or n=3 KO expression values must have exceeded 7 to indicate overall expression in either genotype. A two-tailed t-test which resulted in p-values of less than 0.05 indicated significant differential expression.

qPCR

RNA was isolated from individual mouse retinas at various ages using TRI-reagent (Sigma). cDNA was generated from 400 ng of retinal RNA using random primers and SuperScript III (Life Technologies) according to the manufacturer's instructions. qPCR was performed using SYBRGreen MasterMix (Thermo) and a BioRad CFX96 Real Time System with BioRad C1000 Thermal Cycler using the following program: 15' at 95°C and 40 cycles of 15 sec at 95°C, 30 sec at 56°C, and 30 sec at 72°C. Each sample was normalized to β-Actin primers. The analysis of the qPCR data was performed exactly as in Livak and Schmittgen (Livak and Schmittgen, 2001). Briefly, the difference in average ΔΔC(t) values and the difference plus and minus the standard error of the difference were computed on the C(t) scale. The base-2 antilogs of these three values were computed to obtain values with error bars on the fold change scale. Results were plotted on a logarithmic scale.

Mouse genotyping

Genomic DNA isolation

Tissue used for isolation of genomic DNA included ear clips for live adult mice, which were confirmed using tail samples upon tissue harvest. Tail samples were always employed for embryonic and early postnatal stages. OC1 genomic DNA was isolated by adding 200ul 50mM NaOH to tissue samples and incubating at 95°C for 30 minutes, followed by the addition of 50ul 1M Tris pH 8 and centrifugation for six minutes at 13,300 rpm at 4˚C. OC2 genomic DNA preparation required phenol-chloroform (1:1) extraction followed by ethanol precipitation.

Genotyping PCR

OC1 and OC2 deficient mice were genotyped with primer pairs for the WT and null alleles. OC1-WT: 5'-CAGCACCTCACGCCCACCTC-3', 5'-CAGCCACTTCCACATCCTCCG-3'; OC1-KO: 5'-CTGTGCTCGACGTTGTCACTG-3', 5'-GATCCCCTCAGAAGAACTCGT-3'. OC2-WT: 5'-GCCACGCCGCTGGGCAAC-3', 5'-CAGCTGCCCGGACGTGGC-3'; OC2-KO: 5'-GACCGAGTACAAGCCCACG-3'; 5'-GTCCGCGACCCACACCTT-3'. Reactions containing all four primer pairs for each mouse strain were amplified by 35 cycles of PCR, with the same program for both strains (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, with a 3 minute initial denaturation at 94˚C). OC1 PCR reactions included WT primers at 0.2μM each and null allele primers at 1.6μM each. OC2 PCR reactions included all WT and null allele primers at 0.8μM, as well as 1X MasterAmp (Epicentre).

4. Results

The Math5 transcription factor is expressed during the early stages of mouse retina development in the lineages of many retinal cells, including photoreceptors, amacrine cells, horizontal cells, and ganglion cells (Yang et al., 2003). To identify genes involved in the cell fate determination process of early-born retinal neurons, we isolated single Math5^{+} progenitor cells and identified genes that were strongly correlated with Math5 expression in these cells (Trimarchi et al., 2008). Microarray data revealed that members of the Onecut family of transcription factors (Onecut1 and Onecut2) were present in subsets of Math5^{+} single cells (Figure 1A). To confirm the findings of the single-cell microarrays, as well as to better understand the expression of these factors in the retina, *in situ* hybridization (ISH) was performed for Onecut1 (OC1) (Figure 1B) and Onecut2 (OC2) (Figure 1C) at E12.5, E14.5, E16.5, P0, and adult stages. Both transcription factors were observed in subsets of retinal cells, in an area consistent with expression in retinal progenitor cells and newly generated retinal neurons (Figure 1B,C). To quantify the number of cells that co-expressed Math5 and the Onecut factors, dissociated fluorescent ISH was utilized (Figure 1D,E). At E14.5, a significant percentage (27.2%) of Math5^{+} cells also expressed OC1, while 10.6% of Math5^{+} cells also showed expression of OC2 (Figure 1F). As the retina matured, the expression of both OC1 and OC2 became limited to the apical side of the inner nuclear layer, where the horizontal interneurons are located (Figure 1B,C). In addition, OC1 expression was observed in a subset of cells

in the GCL (Figure 1B). Interestingly, only OC1 expression was observed in the GCL and not OC2, whereas both proteins were previously reported to be in the GCL by antibody staining (Wu et al., 2012). This may reflect a difference in mRNA and protein expression. Otherwise, the timing and location of expression of Onecut1 and Onecut2 is consistent with a recent publication (Wu et al., 2012) and suggests a possible role for the Onecut transcription factors in regulating retinal development. With these data in mind, we obtained OC1 and OC2-deficient mice (Clotman, et al., 2005) and determined how loss of these transcription factors affected retinal cell populations.

Retinal phenotypes in the OC1-deficient mouse

Previous studies have demonstrated that OC1 is a critical transcription factor in liver development (Clotman, et al., 2002). Due to this important function, most OC1-KO mice die either at birth or in the immediate postnatal period. Despite the high mortality rate resulting from complete loss of OC1, we were able to obtain a few mature, postnatal day (P)21 OC1-KO mice. Since the effects of OC1 deficiency on retinal development had not previously been studied in this particular mouse line, we examined whether mature retinal cell populations were present in the absence of OC1. To specifically determine any deficits in populations of retinal cells, P21 OC1-KO retinas were surveyed using immunohistochemistry with antibodies that mark each subset of retinal cells (Figure 2). Compared to wildtype littermate control animals (Figure 2A-E), populations of rod photoreceptors (Figure 2A'), amacrine cells (Figure 2B'), bipolar cells (Figure 2C',D'), and Muller glia (Figure 2E') were

unchanged in adult OC1-KO mice. OC1 has previously been shown to play an important role in the development of horizontal cells using a conditional knockout mouse model (Wu et al., 2013). Consistent with this report, a significantly decreased horizontal cell population was readily apparent in our P21 OC1-deficient mice (Figure 2F,F').

Retinal phenotypes in the OC2 deficient mouse

We observed that OC2 is expressed at developmental timepoints and in cell populations reminiscent of its family member, OC1 (Figure 1). Therefore, we next explored the consequences resulting from the loss of OC2 during retinal development. To determine whether the development of retinal cell populations was disrupted by loss of OC2, adult (P21) retinal cell populations of OC2-deficient mice were surveyed by immunohistochemistry using a similar panel of antibodies as in the OC1-deficient mouse retinas (Figure 3). No changes were observed in populations of rod photoreceptors (as marked by αRho4d2, Figure 3A,A'), amacrine interneurons (αPax6, Figure 3B,B'), bipolar cells (αIsl1, Figure 3C,C'; αChx10, Figure 3D,D'), or Muller glia (αGS, Figure 3E,E'). However, horizontal cell populations were significantly decreased in OC2-KO, to a similar degree as observed in the OC1-KO (Figure 3F,F'). To ensure there were not any small, but significant differences in these cell types, cell counts were performed on sections from n=3 different wildtype and OC2-KO mice. No significant differences were observed for any cell types except the horizontal cells ($P<0.005$, Figure 3G). To further confirm the results obtained through immunohistochemical analysis, we performed *in situ* hybridizations on

cryosections from P21 OC2-KO retinas. We did not observe any significant differences between OC2-KO animals and their wildtype littermates in either the rod or cone photoreceptor populations (Supplementary Figure 1 (SF1) A-C'), bipolar cells (SF1D,D'), GABAergic (GAD1 – SF1E,E') and glycinergic (SLC6A9 – SF1F,F') amacrine interneurons (SF1G,G',H,H'), ganglion cells (SF1I,I'), and Muller glia (SF1J,J'). However, ISH did reveal a decrease in horizontal cells (SF1K,K',L,L').

To gain perspective on the overall patterning of horizontal cell loss, whole, flat-mounted retinas were stained using an antibody against the horizontal cell marker Calbindin-28k (Calb28k) (Figure 4). Whole mount staining showed a significant loss of the horizontal cell population across the entire retinas of both the OC1 (Figure 4A,A') and OC2-deficient mice (Figure 4B,B'). In P21 OC2-deficient retinas, horizontal cells were decreased to about 20% of the levels seen in wildtype littermates ($n=3, p=2e^{-7}$). Given the difficulty of obtaining OC1 mice at maturity we were only able to quantify one animal, but the horizontal cells were decreased by 35%. These observations demonstrate that loss of either Onecut1 or Onecut2 interferes with either the generation or maintenance of horizontal cells in the murine retina.

In a previous study, Onecut1 deficient retinas were found to exhibit degeneration of the photoreceptor layer (ONL) by 8 months of age (Wu et al., 2013). Therefore, we examined retinas of OC2-deficient mice for possible ONL degeneration at two different ages (Supplementary Figure 2 [SF2]). Staining for Calbindin-28k indicated that the decrease in horizontal cells in OC2-KO mice

compared to WT mice was maintained well into maturity (SF2). However, antibody staining for rod photoreceptors in the OC2-KO appeared grossly normal at 12 months of age when compared to a wildtype littermate (SF2A,A'). Conversely, a 16-month old OC2-deficient mouse displayed a thinner layer of rhodopsin staining compared to a WT age-matched littermate (SF2B,B'). These results indicate that although the horizontal cells are similarly decreased in both Onecut1 and Onecut2 deficient mice, the photoreceptor degeneration is much slower and less pronounced in the OC2-KO mouse compared to the OC1-KO.

We have observed that mature mouse retinas are either unable to generate or maintain wildtype numbers of horizontal cell populations in the absence of either OC1 or OC2. To better understand the developmental stage when this phenotype becomes apparent, we investigated OC2 deficient retinas at various stages of maturity, starting with P5-10 (Figure 5). At these stages, nearly all retinal cells, including horizontal cells, are postmitotic (Young, 1985a). Immunohistochemical analysis showed that at P5, the development of other retinal cells, including the late-developing rod photoreceptors (αRho, Figure 5A,A') and bipolar cells (αChx10, Figure B,B') appear normal. However, postnatal retinas lacking Onecut2 already show a marked decrease in horizontal cells both at P5 (Figure 5C,C') and at P10 (Figure 5D,D'). These results suggest that the loss of horizontal cells in OC2-deficient retinas may not be due to a lack of horizontal cell maintenance, at least in the postnatal stages of development.

It is apparent that horizontal cell populations are disrupted before retinal maturation is completed in OC2-KO retinas. However, it is unclear whether the progenitor cells that would normally become horizontal cells undergo a fate change and become other retinal cell types, or if they differentiate normally into horizontal cells only to die in the absence of OC2. Horizontal cells are among the earliest born cell types in the retina, so a cell-fate change might be predicted to affect the population sizes of other early-born neurons. To examine whether a cell fate change was apparent in early retinal differentiation, ISH was performed at E16.5 (Supplementary Figure 3 [SF3]). A high percentage of early-born retinal cells including cone photoreceptors, some amacrine interneurons, and ganglion cells were generated by this late embryonic stage. There were no significant differences in progenitor cells at E18.5 as visualized by Chx10 (SF3A,A'), photoreceptor precursor cells (Prdm1, SF3B,B'; Otx2, SF3C,C'; Gnb3, SF3D,D'), amacrine cell precursors (Tcfap2b, SF3E,E'), and developing ganglion cells (Sncg, SF3F,F') in the absence of OC2.

Although gross morphological changes in early-born retinal populations are not apparent at E16.5 in OC2-KO mice, we were also interested in visualizing the cell types generated in the early stages of retinogenesis in OC1-KO mice. We performed *in situ* hybridization at E16.5, a timepoint when horizontal cells, ganglion cells, cone photoreceptors, and amacrine cells are being actively produced (Supplementary Figure 4 [SF4]). As in the OC2-KOs, no changes were identified during mid-embryonic stages in the developing photoreceptors as visualized using probes to Prdm1, Gnat2, and Gnb3, (SF4A,A'-C,C'). Additionally, no significant changes were

noted in amacrine cells (Tcfap2b, SF4D,D') or ganglion cells (Sncg, SF4E,E'). Horizontal cells are an extremely small population of neurons that are generated over multiple days during murine retinogenesis (Cepko, 1996) and are, therefore, difficult to track over the span of their generation. We have not observed any changes in other retina cell populations or indications of increased retinal cell death. However, the effect of the loss of cells or fate-switch in such a small population may not generate statistically significant changes in cell death or cell-fate change at any single point over the developmental timeline.

Global gene expression changes in Onecut2-deficient mouse retina

To assess global changes in gene expression that were not immediately apparent from our visual inspection of retinal cell populations, we performed microarray analysis on adult OC2 retinas (data available on NCBI GEO: reference number GSE57918). We first examined genes that were downregulated in adult OC2-KO retinas (Supplemental table 1). Our prediction was that genes with lower expression would point to any additional cellular deficiencies in the OC2-KO mice. Consistent with our previous observations regarding a significant deficit in horizontal cells in these mice, the genes Calb1 and Septin4 (Blackshaw et al., 2004) were significantly downregulated (Supp. Table 1). Surprisingly, the signals for two bipolar cell genes, Pcp2 and Car8 (Kim et al., 2008), were also significantly downregulated, indicating a subtle bipolar cell phenotype in the OC2-KO mice.

The LIM1 class transcription factor Lhx1 is specifically expressed in differentiating and mature horizontal cells (Poché et al., 2007). We examined our microarray data and found that while the expression of Lhx1 was decreased, it did not reach significance ($p=0.159$). To ascertain whether Lhx1 was in fact downregulated in OC2-KO retinas, we performed qPCR and found that Lhx1 expression was significantly decreased in the OC2-KO adult retina (Figure 6A). To further pinpoint when this downregulation occurs, we performed qPCR for Lhx1 on WT and OC2-KO retinas at E16.5. At this timepoint, we also observed that Lhx1 expression was significantly lower in the OC2-KO than in the WT ($p<0.005$, Figure 6A), suggesting that horizontal cells are decreased in these mice from very early on in development. Interestingly, our data suggests that this regulation of Lhx1 by the Onecut factors may be dosage dependent. Removal of just a single copy of either OC1 or OC2 (data not shown) reduced the amount of Lhx1 present. This result was consistent, but not high enough to reach statistical significance. Together, these microarray and qPCR results point to an early defect in horizontal cells and a possible bipolar cell phenotype in the OC2 deficient mice.

Next, we examined genes that were upregulated in the absence of OC2 (Supplemental Table 2). Curiously, OC2 was one of the most highly upregulated genes in OC2-KO retinas as compared to their WT littermates. To understand this conundrum, we investigated the location of the sequences used on the microarray and ISH-probes and found that they were located at the 3' end of the Onecut2 gene. To see if this upregulation was consistent throughout the entire sequence, we targeted the 5' coding sequence of the Onecut2 gene by qPCR and found it to be

virtually undetectable (data not shown). Additionally, OC2 protein as visualized through immunohistochemistry was not observed in the OC2-KO retinas (data not shown). These observations suggest that the remaining leftover OC2 sequence in the OC2-KO mouse is upregulated at the RNA level, but that no functional protein is produced.

Another highly upregulated gene observed in OC2-KO retinas was glial fibrillary acidic protein (GFAP), a marker of activated gliosis (Lewis and Fisher, 2003). To confirm the upregulation of GFAP, qPCR was performed on OC2-deficient retinas. A twofold upregulation of GFAP was observed (n=7 WT, n=9 KO animals; p=0.002; Figure 6B). Antibody staining for GFAP revealed that the processes of Muller glia have upregulated the protein in OC2-KO retinas when compared with the WT littermates (Figure 6C). To get a greater sense of other changes present in the KO retinas, especially those related to a possible stress response, we explored changes in other genes identified to be upregulated in models of retinal disease. Indeed, further investigation of markers of retinal stress indicated that adult OC2 retinas overexpress numerous genes implicated in stress or injury-related contexts (Rattner, 2005; Samardzija et al., 2012; Swiderski et al., 2007; Templeton et al., 2013), such as Edn2, C4b, and Cebpd, in both microarray and follow-up qPCR (Figure 6B). In addition, CD44, a Muller glia expressed gene that is increased upon retinal degeneration (Chaitin et al., 1996), is upregulated in OC2-KO retina microarrays (Supplemental Table 2). Despite the normal rhodopsin staining observed in OC2-KO retinas, the upregulation of these stress response and gliosis-

associated genes suggest that retina health is compromised in the absence of OC2 and/or horizontal cells.

To ascertain any developmental phenotypes in the OC1-KO retinas, microarrays were performed on retinas isolated from E14.5 mice (data available on NCBI GEO: reference number GSE57917). Among the genes that were consistently upregulated, two of them stood out. Gephyrin and Sox4 were significantly upregulated in the E14.5 OC1-KO retinas (Supplemental Table 3). Given the expression of these genes in ganglion and amacrine cells (Grünert and Wässle, 1993; Jiang et al., 2013), their upregulation indicates a subtle phenotype in these cells. One interesting gene that was observed to have a higher, but not quite statistically significant, expression in the E14.5 OC1-KO versus WT was melanopsin (Opn4). Opn4 encodes a photopigment protein that is expressed in a small subset of intrinsically photosensitive retinal ganglion cells (Schmidt et al., 2011). While much research has been done on the function of these cells, very little is known about how these cells are generated and how the Opn4 gene is regulated. Since the differential expression of Opn4 only approached significance ($p=0.08$), we examined the expression of Opn4 by qPCR in WT and OC1-KO retinas at E14.5. By this assay we observed a statistically significant upregulation of Opn4 (Figure 6D). Surprisingly, when we examined OC1-KO retinas at slightly later timepoints (E16.5 and E17.5) we no longer observed this upregulation of Opn4 (data not shown); this suggests that other factors may be able to compensate for the loss of OC1. These data identify the Onecut1 transcription factor as a possible repressor of Opn4 expression.

We also examined the genes that were downregulated in E14.5 OC1-KO retinas when compared to WT littermates (Supplemental Table 4). While most of the genes that were downregulated have unknown retinal functions, two genes, Irx4 and Scratch2, stood out. These two genes have been associated with developing retinal ganglion cells (Jin et al., 2003; Trimarchi et al., 2007), suggesting that there may be subtle defects in retinal ganglion cell development or differentiation in the OC1-KO retinas that warrant further investigation.

5. Discussion

In this study, our goal was to better understand the factors that contribute to neurogenesis in the developing murine retina. By profiling the transcriptomes of individual cells expressing Math5, a transcription factor integral to the early stages of retinal development (Brown et al., 2001; Kay et al., 2001; Wang, 2001), we identified two genes, Onecut1 (OC1) and Onecut2 (OC2), which were also expressed in the developing mouse retina. Specifically, we found that both OC1 and OC2 were expressed in patterns similar to Math5 in the embryonic retina and later their expression became more confined to mature horizontal cells. An examination of mature retinas from mice deficient for either OC1 or OC2 revealed that loss of either factor led to a marked decrease in adult horizontal cell populations as seen through immunohistochemistry, *in situ* hybridization, microarrays, and qPCR analysis. Microarray analysis of OC2-deficient retinas also revealed an upregulation of stress-

related genes, such as GFAP, indicative of retinal stress and reactive gliosis. However, significant degeneration of the ONL was not observed until 16 months of age in the OC2-KO mice. Additionally, our microarray profiling has revealed potential phenotypes in subsets of other retinal cell types, which were not observed either in our bulk population antibody staining or in a previous study of a conditional Onecut1 retinal knockout (Wu et al., 2013). Overall, our results indicate that both OC1 and OC2 play an important role in the development of horizontal cell populations within the retina, and that the loss of these transcription factors compromises the overall health of the mature retina.

OC1 and OC2 appear to share overlapping roles in the development of the retina. Loss of either gene alone led to a decrease in horizontal cells, although neither deficiency on its own led to a complete lack of these cells. This suggests some amount of redundancy in the functionality of these Onecut family members during retinal development. Our results are consistent with the fact that OC1 and OC2 share a 92% amino acid similarity in their DNA binding domains (Jacquemin et al., 2003) and that they have been shown to bind to the same consensus DNA binding site (Iyaguchi et al., 2007). Although embryonic lethality precluded an in-depth analysis of this redundancy, as we were unable to recover viable double-KO mice, we were able to examine mice lacking three of the four OC1 and OC2 alleles by qPCR. These analyses indicated that the changes in expression in the transcription factor Lhx1 are more severe as additional alleles of the Onecut family genes are removed (data not shown). Although we were unable to obtain enough animals to perform a rigorous statistical test on this data, our consistent results on those

available do suggest a possible dosage-dependent model for Onecut regulation of Lhx1.

The expression of Onecut1 and Onecut2 has been previously examined during retinal development (Wu et al., 2012). As in our single-cell studies, the previous association of OC1 and OC2 with Math5 in early retinal development suggested that these genes play a role in the cell-fate determination of early-developing retinal cells. Our findings agree with previous results that OC1 is critical for the development of horizontal cells (Wu et al., 2013). We have expanded our analysis to that of the related family member, OC2, which appears to have a similar function in the development of horizontal cells in the retina. Surprisingly, even though OC2 was expressed in fewer horizontal cell progenitors (Wu et al., 2012), it led to a very similar reduction in the number of horizontal cells in adult KO mice. Studies of mice where both OC1 and OC2 are conditionally removed from the retina will allow for a further dissection of the relative contribution of each family member.

Although loss of either OC1 or OC2 affects the developing retina in similar ways, leading to a drastic loss of horizontal cells, our study also shows that the absence of either of these factors leads to additional phenotypes in the retina. Our microarray analyses allow for an unbiased examination of numerous transcriptomic changes upon loss of these two family members. For instance, transcriptome analysis of E14.5 OC1-KO mice revealed that the expression of Opn4, the photopigment seen in intrinsically-photosensitive ganglion cells, is increased upon

OC1 loss. It is curious that the upregulation of Opn4 is transient and the identification of other factors that may compensate for the loss of OC1 warrants future study. The increase in Opn4, along with the observed upregulation of gephyrin and Sox4 and downregulation of Irx4 and Scratch2, suggests that OC1 may play a previously unrecognized role in a subset of ganglion cells (or other retinal cells) during retinal development. Additionally, the upregulation of gephyrin could point to an increase in amacrine cells, as was predicted by the fact that in the absence of OC1, horizontal cell precursors would be expressing the transcription factor Ptf1a (Wu et al., 2013).

Our findings in the OC2-KO mouse contrast with previous findings in the OC1-conditional KO mouse that indicate retinal degeneration begins as early as 5 months of age in OC1-deficient retinas and continues to progress. Although our OC2-KO mice do show an upregulation of genes associated with retinal stress during early adulthood (Figure 6B,C) and a downregulation of some bipolar expressed genes, we do not observe distinctive signs of retinal degeneration until well past one year of age (SF2). This lack of degeneration may relate to the viability of our full knockouts in comparison to the conditional knockout mice previously described (Wu et al., 2013). As the Onecut transcription factors play integral roles in liver, pancreatic, and immune function (Clotman et al., 2002; Furuno et al., 2008; Jacquemin et al., 2003), a substantial number of our OC2-KO mice and almost all of our OC1-KO mice die soon after birth. Therefore, it may be that those mice that survive to advanced age have activated some compensatory mechanisms or contain some genetic modifiers that allow for a more normal development upon Onecut loss.

Previously we had found that the cone marker thyroid hormone receptor beta 2 (Thrb) was downregulated in the OC1-KO mice (Emerson et al., 2013). However, Thrb appeared at normal levels by qPCR in the embryonic retinas of the OC2-deficent mice (data not shown). Moreover, we found that other markers of cone photoreceptors, including Gnat2 and Pde6c, appeared normal in the OC2-KO mouse retina, further indicating that cone photoreceptors are not affected in these mice. Surprisingly, we also found that Gnat2 and Prdm1 appeared normal in the embryonic retina of the OC1-KO mouse, despite the downregulation of Thrb. These data may point to a compensatory mechanism operating during retinal development to replace Onecut1 in photoreceptor precursor cells. It may be that other Onecut family members are responsible for this mechanism, but lethality prevents the analysis of compound mutant mice in our background. Further work utilizing additional conditional mouse knockouts will be required to more accurately define the overlapping and distinct roles that the Onecut transcription factors play in retinal development.

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7. References

Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W.P., Weber, G., Lee, K., Fraioli, R.E., Cho, S.-H., et al. (2004). Genomic Analysis of Mouse Retinal Development. PLoS Biol. *2*, e247.

Brown, N.L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Dev. Camb. Engl. *128*, 2497–2508.

Brzezinski, J.A., 4th, Prasov, L., and Glaser, T. (2012). Math5 defines the ganglion cell competence state in a subpopulation of retinal progenitor cells exiting the cell cycle. Dev. Biol. *365*, 395–413.

Cepko, C.L. (1996). The patterning and onset of opsin expression in vertebrate retinae. Curr. Opin. Neurobiol. *6*, 542–546.

Chaitin, M.H., Ankrum, M.T., and Wortham, H.S. (1996). Distribution of CD44 in the retina during development and the rds degeneration. Brain Res. Dev. Brain Res. *94*, 92–98.

Chen, Z., Li, X., and Desplan, C. (2012). Deterministic or Stochastic Choices in Retinal Neuron Specification. Neuron *75*, 739–742.

Cherry, T.J., Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2009). Development and diversification of retinal amacrine interneurons at single cell resolution. Proc. Natl. Acad. Sci. *106*, 9495–9500.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. (2002). The onecut transcription

factor HNF6 is required for normal development of the biliary tract. Dev. Camb. Engl. *129*, 1819–1828.

Edlund, T., and Jessell, T.M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell *96*, 211–224.

Emerson, M.M., Surzenko, N., Goetz, J.J., Trimarchi, J., and Cepko, C.L. (2013). Otx2 and Onecut1 Promote the Fates of Cone Photoreceptors and Horizontal Cells and Repress Rod Photoreceptors. Dev. Cell *26*, 59–72.

Feng, L., Xie, Z., Ding, Q., Xie, X., Libby, R.T., and Gan, L. (2010). MATH5 controls the acquisition of multiple retinal cell fates. Mol. Brain *3*, 36.

Furuno, K., Ikeda, K., Hamano, S., Fukuyama, K., Sonoda, M., Hara, T., Sasazuki, T., and Yamamoto, K. (2008). Onecut transcription factor OC2 is a direct target of T-bet in type-1 T-helper cells. Genes Immun. *9*, 302–308.

Goetz, J.J., Farris, C., Chowdhury, R., and Trimarchi, J.M. (2014). Making of a Retinal Cell. In International Review of Cell and Molecular Biology, (Elsevier), pp. 273–321.

Grünert, U., and Wässle, H. (1993). Immunocytochemical localization of glycine receptors in the mammalian retina. J. Comp. Neurol. *335*, 523–537.

He, J., Zhang, G., Almeida, A.D., Cayouette, M., Simons, B.D., and Harris, W.A. (2012). How Variable Clones Build an Invariant Retina. Neuron *75*, 786–798.

Isshiki, T., Pearson, B., Holbrook, S., and Doe, C.Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell *106*, 511–521.

Iyaguchi, D., Yao, M., Watanabe, N., Nishihira, J., and Tanaka, I. (2007). DNA recognition mechanism of the ONECUT homeodomain of transcription factor HNF-6. Struct. Lond. Engl. 1993 *15*, 75–83.

Jacquemin, P., Lemaigre, F.P., and Rousseau, G.G. (2003). The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. Dev. Biol. *258*, 105–116.

Jiang, Y., Ding, Q., Xie, X., Libby, R.T., Lefebvre, V., and Gan, L. (2013). Transcription Factors SOX4 and SOX11 Function Redundantly to Regulate the Development of Mouse Retinal Ganglion Cells. J. Biol. Chem. *288*, 18429–18438.

Jin, Z., Zhang, J., Klar, A., Chédotal, A., Rao, Y., Cepko, C.L., and Bao, Z.-Z. (2003). Irx4-mediated regulation of Slit1 expression contributes to the definition of early axonal paths inside the retina. Dev. Camb. Engl. *130*, 1037–1048.

Kay, J.N., Finger-Baier, K.C., Roeser, T., Staub, W., and Baier, H. (2001). Retinal Ganglion Cell Genesis Requires lakritz, a Zebrafish atonal Homolog. Neuron *30*, 725–736.

Kim, D.S., Ross, S.E., Trimarchi, J.M., Aach, J., Greenberg, M.E., and Cepko, C.L. (2008). Identification of molecular markers of bipolar cells in the murine retina. J. Comp. Neurol. *507*, 1795–1810.

Lemaigre, F.P., Durviaux, S.M., Truong, O., Lannoy, V.J., Hsuan, J.J., and Rousseau, G.G. (1996). Hepatocyte nuclear factor 6, a transcription factor that contains a novel type of homeodomain and a single cut domain. Proc. Natl. Acad. Sci. U. S. A. *93*, 9460–9464.

Lewis, G.P., and Fisher, S.K. (2003). Up-regulation of glial fibrillary acidic protein in response to retinal injury: its potential role in glial remodeling and a comparison to vimentin expression. Int. Rev. Cytol. *230*, 263–290.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods *25*, 402-408.

Livesey, F.J., and Cepko, C.L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. *2*, 109–118.

Masland, R.H. (2001). The fundamental plan of the retina. Nat. Neurosci. *4*, 877–886.

Molday, R.S., and MacKenzie, D. (1983). Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry (Mosc.) *22*, 653–660.

Morrow, E.M., Chen, C.-M.A., and Cepko, C.L. (2008). Temporal order of bipolar cell genesis in the neural retina. Neural Develop. *3*, 2.

Poché, R.A., Kwan, K.M., Raven, M.A., Furuta, Y., Reese, B.E., and Behringer, R.R. (2007). Lim1 is essential for the correct laminar positioning of retinal horizontal cells. J. Neurosci. Off. J. Soc. Neurosci. *27*, 14099–14107.

Rattner, A. (2005). The Genomic Response to Retinal Disease and Injury: Evidence for Endothelin Signaling from Photoreceptors to Glia. J. Neurosci. *25*, 4540–4549.

Samardzija, M., Wariwoda, H., Imsand, C., Huber, P., Heynen, S.R., Gubler, A., and Grimm, C. (2012). Activation of survival pathways in the degenerating retina of rd10 mice. Exp. Eye Res. *99*, 17–26.

Schmidt, T.M., Do, M.T.H., Dacey, D., Lucas, R., Hattar, S., and Matynia, A. (2011). Melanopsin-Positive Intrinsically Photosensitive Retinal Ganglion Cells: From Form to Function. J. Neurosci. *31*, 16094–16101.

Sidman, R. (1961). Histogenesis of mouse retina studied with thymidine-H3. In The Structure of the Eye, G. Smelser, ed. (New York: Academic Press), pp. 487–506.

Skowronska-Krawczyk, D., Chiodini, F., Ebeling, M., Alliod, C., Kundzewicz, A., Castro, D., Ballivet, M., Guillemot, F., Matter-Sadzinski, L., and Matter, J.-M. (2009). Conserved regulatory sequences in Atoh7 mediate non-conserved regulatory responses in retina ontogenesis. Development *136*, 3767–3777.

Swiderski, R.E., Nishimura, D.Y., Mullins, R.F., Olvera, M.A., Ross, J.L., Huang, J., Stone, E.M., and Sheffield, V.C. (2007). Gene Expression Analysis of Photoreceptor Cell Loss in Bbs4-Knockout Mice Reveals an Early Stress Gene Response and Photoreceptor Cell Damage. Invest. Ophthalmol. Vis. Sci. *48*, 3329–3340.

Templeton, J.P., Freeman, N.E., Nickerson, J.M., Jablonski, M.M., Rex, T.S., Williams, R.W., and Geisert, E.E. (2013). Innate Immune Network in the Retina Activated by Optic Nerve Crush. Invest. Ophthalmol. Vis. Sci. *54*, 2599–2606.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual Retinal Progenitor Cells Display Extensive Heterogeneity of Gene Expression. PLoS ONE *3*, e1588.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Voinescu, P.E., Kay, J.N., and Sanes, J.R. (2009). Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. J. Comp. Neurol. *517*, 737–750.

Wang, S.W. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev. *15*, 24–29.

Wu, F., Sapkota, D., Li, R., and Mu, X. (2012). Onecut 1 and Onecut 2 are potential regulators of mouse retinal development. J. Comp. Neurol. *520*, 952–969.

Wu, F., Li, R., Umino, Y., Kaczynski, T.J., Sapkota, D., Li, S., Xiang, M., Fliesler, S.J., Sherry, D.M., Gannon, M., et al. (2013). Onecut1 Is Essential for Horizontal Cell Genesis and Retinal Integrity. J. Neurosci. *33*, 13053–13065.

Yang, Z., Ding, K., Pan, L., Deng, M., and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. *264*, 240–254.

Young, R.W. (1985a). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

Young, R.W. (1985b). Cell proliferation during postnatal development of the retina in the mouse. Dev. Brain Res. *21*, 229–239.

8. Figures and legends

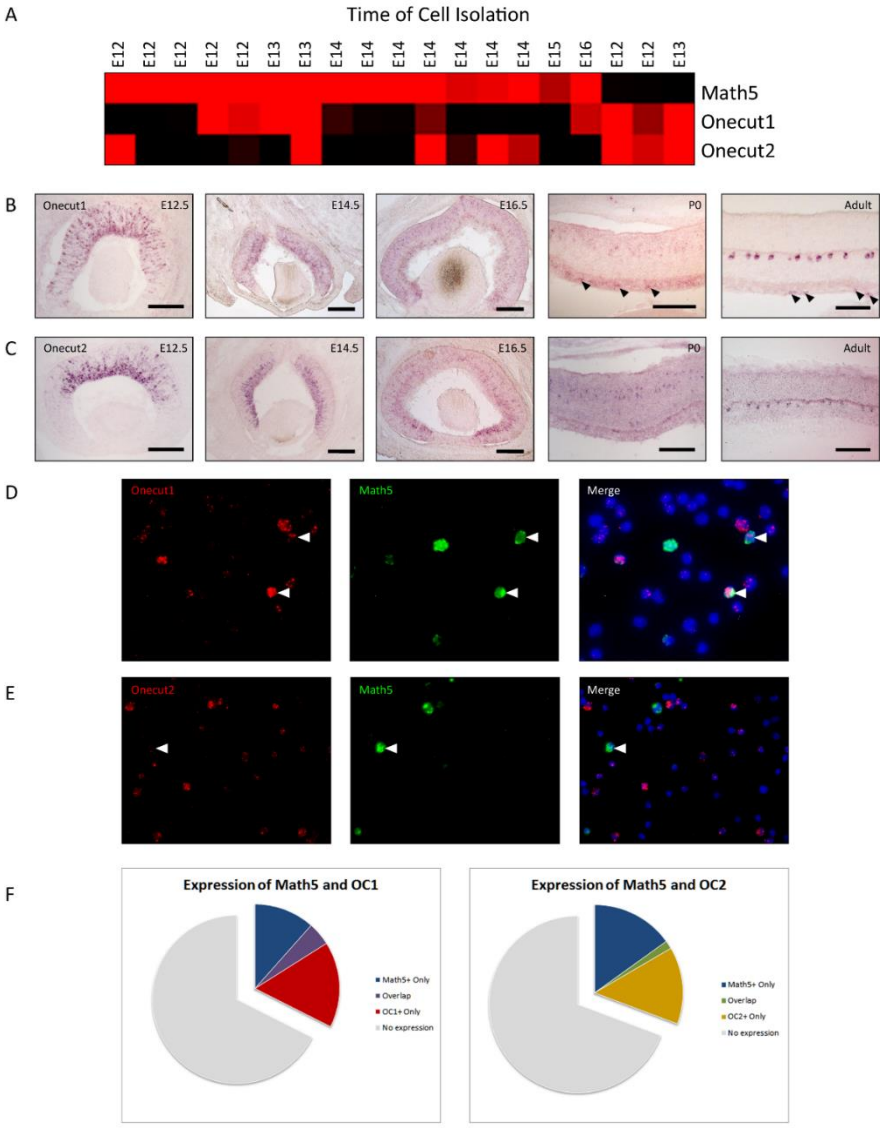


Figure 1. Expression of Onecut1 and Onecut2 in the developing murine retina.

The expression of Onecut transcription factors was analyzed throughout retinal development (A). A heatmap indicating the genes (rows) expressed in isolated single retinal progenitor cells (columns) at various stages of development, from embryonic day (E)12.5 to E16.5. Increased expression of a gene in a given cell is indicated in shades of red, while the absence of expression is indicated with a black square. Expression patterns of Onecut1 (B) and Onecut2 (C) mRNAs were determined via in situ hybridization at various stages of retinal development and in

the adult retina. Arrowheads indicate expression in the ganglion cell layer. Adult scale bars represent 100 μm; all others 200 μm. (D,E) Dissociated cell in situ hybridization was performed at E14.5 using a probe targeting either Onecut1 or Onecut2 and Math5. Arrowheads indicate overlapping Math5 and Onecut family member expression. (F) Quantification of dissociated retinal cells expressing Math5, Onecut1, and Onecut2.

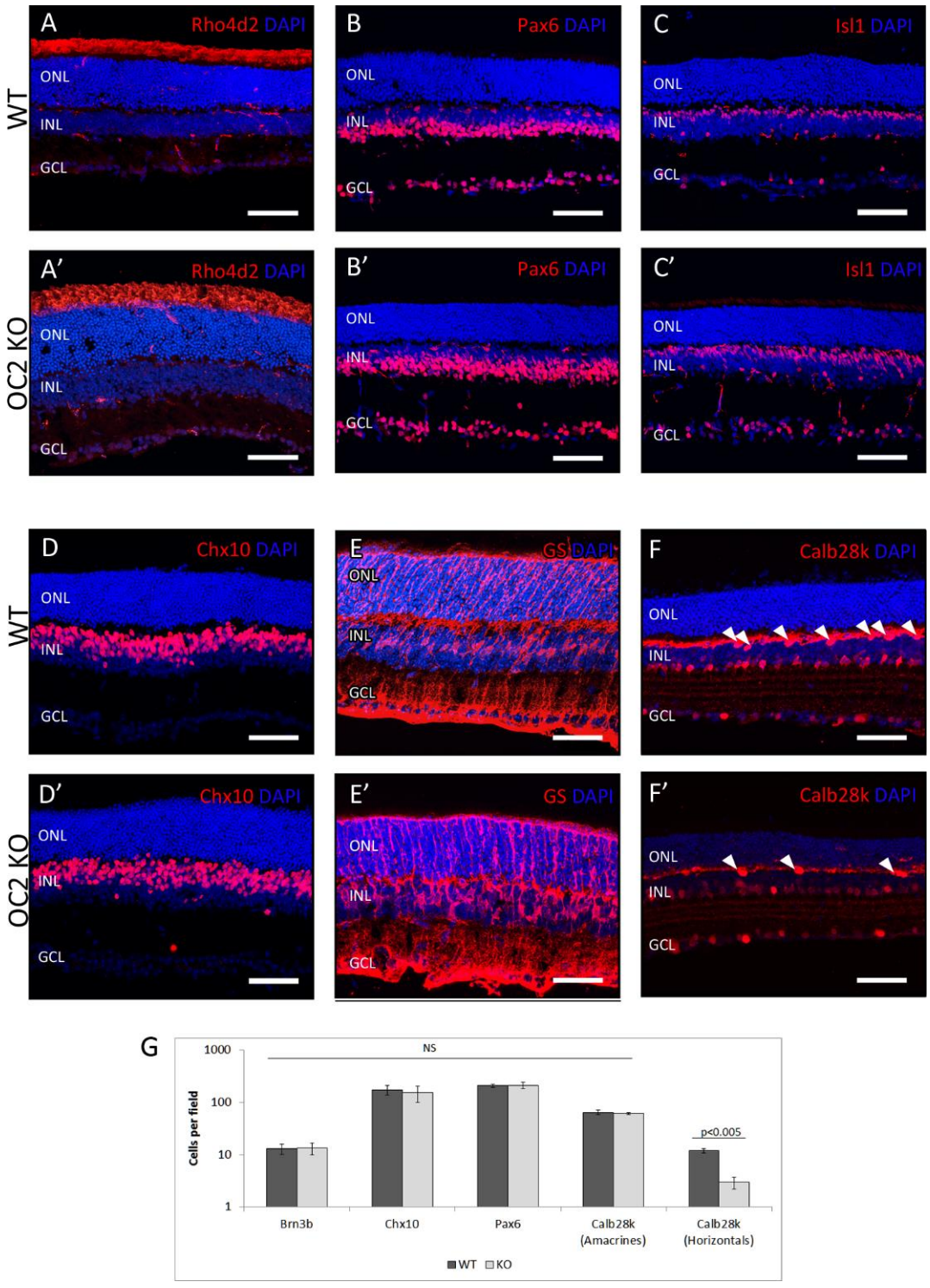


Figure 2. Immunohistochemistry of Adult OC1 Knockout Retinas

Changes in mature retinal cell populations resulting from the loss of OC1 were examined using immunohistochemistry. Most retinal populations were unchanged

between wildtype and OC1-KO littermates, including rod photoreceptors (A,A'), amacrine cells (B,B',C,C'), bipolar cells (D,D'), and Muller glia (E,E'). However, anti-Calb28k-staining shows a decrease in horizontal cells in OC1-KO retinas as compared to a wildtype littermate (F,F'). Arrowheads indicate horizontal cell nuclei. Scale bars represent 100 μm.

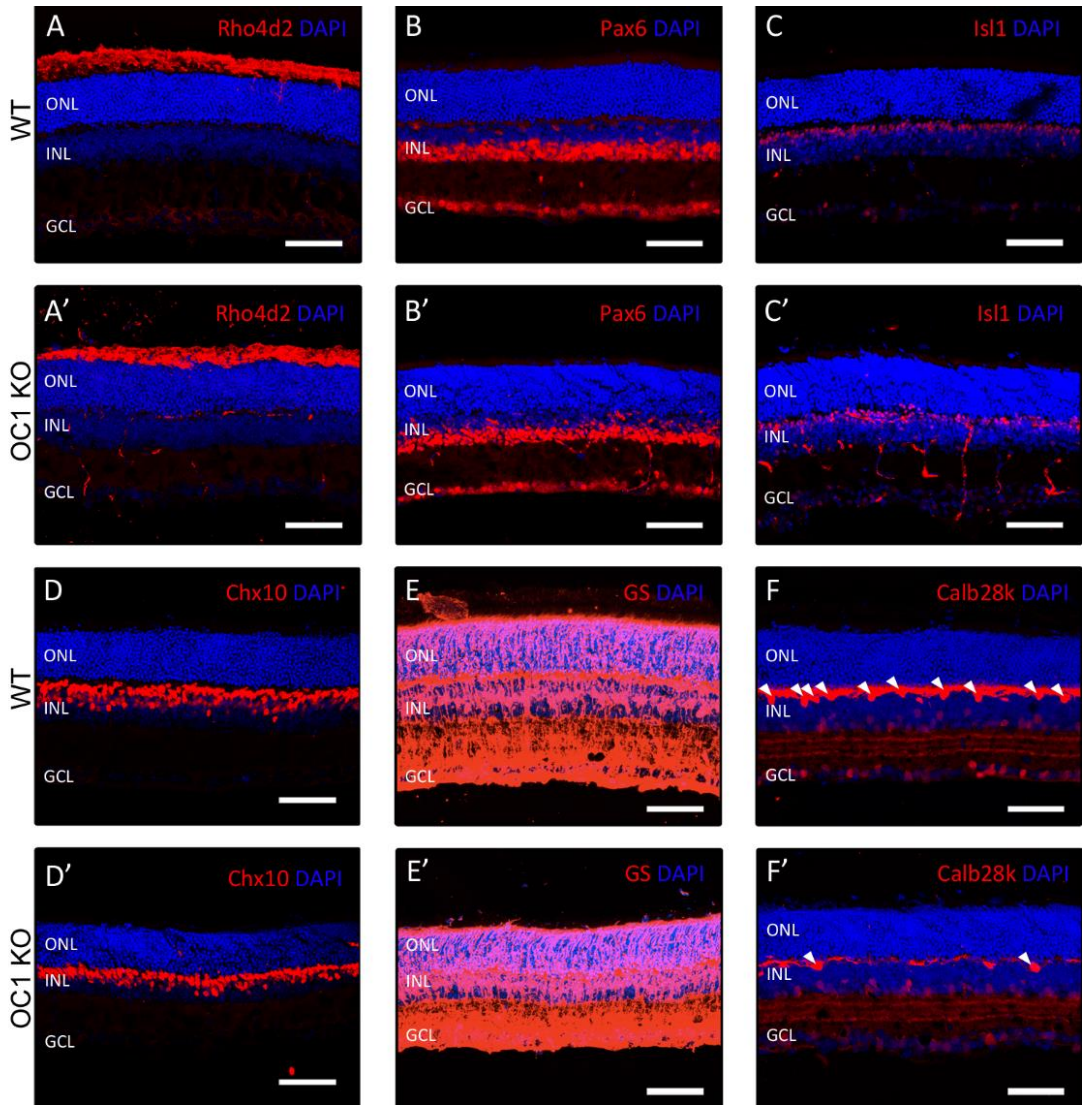


Figure 3. Immunohistochemistry of Adult OC2 Knockout Retinas

Changes in mature retinal cell populations resulting from the loss of OC2 were examined using immunohistochemistry. Rod photoreceptors (A,A'), amacrine cells (B,B',C,C'), bipolar cells (D,D'), and Muller glia (E,E') were unchanged, whereas the horizontal cell population was greatly decreased (F,F'; arrowheads indicate horizontal cell bodies). Scale bars represent 100 µm. (G) The results of the immunohistochemical analyses were quantified for identically-sized fields of cryosectioned retinal tissue. Although staining for Brn3b, Chx10, Pax6, and the Calb28k that marks amacrine cell bodies were unchanged, horizontal cell bodies marked by Calb28k were significantly decreased in OC2-KO retinas ($n=3, p<0.005$).

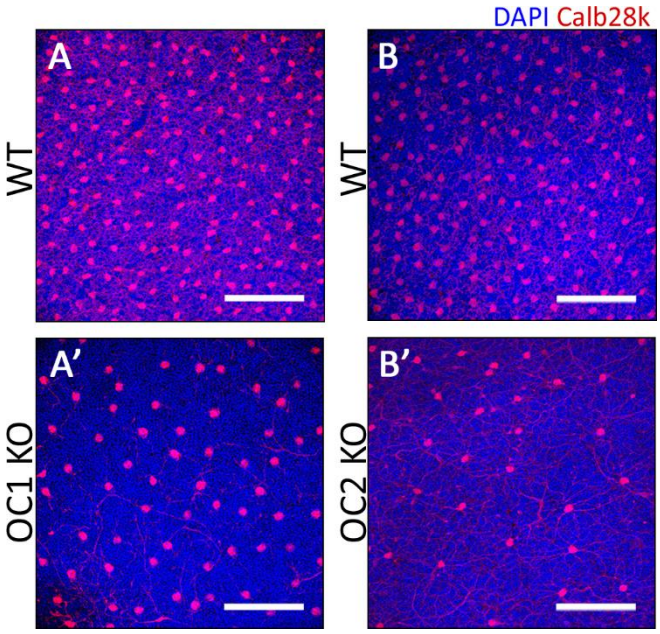


Figure 4. Assessment of horizontal cells by flat mount retina staining

To better understand the distribution of horizontal cell loss in the OC1 and OC2-KO retinas, immunohistochemistry was performed using an anti-Calbindin 28k antibody on age-matched WT (A,B), OC1-KO (A'), and OC2-KO (B') retinas. Scale bars represent 100 μm.

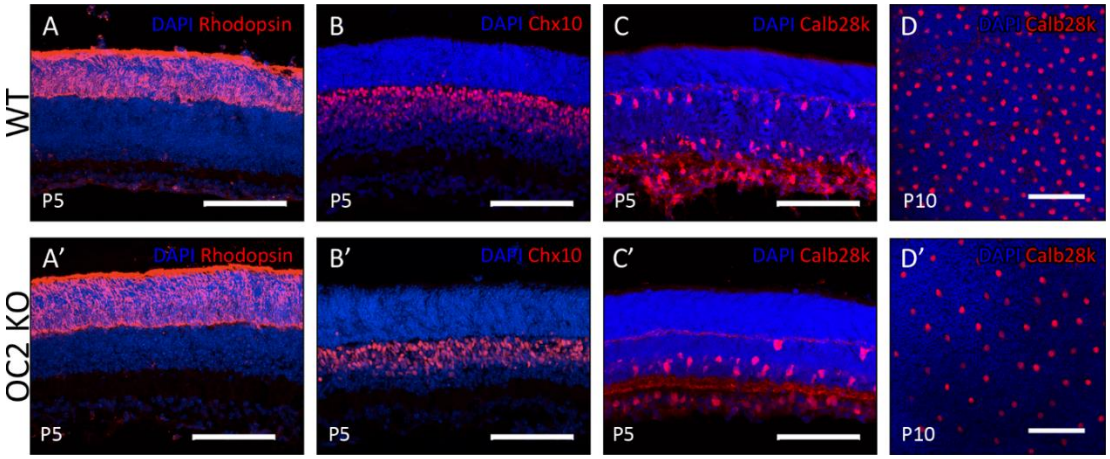


Figure 5. Examination of early postnatal stages in OC2-KO retinas.

To determine whether this decrease in horizontal cells was confined to the fully mature retina, or was present earlier in development, immunohistochemistry was performed on the early postnatal retina. Age-matched animals were processed for either retinal sections (P5) or flatmounts (P10) and stained with an anti-Rhodopsin antibody (A,A'), an anti-Chx10 antibody (B,B'), or an anti-Calbindin 28k antibody (C,C',D,D'). Scale bars represent 100 μm.

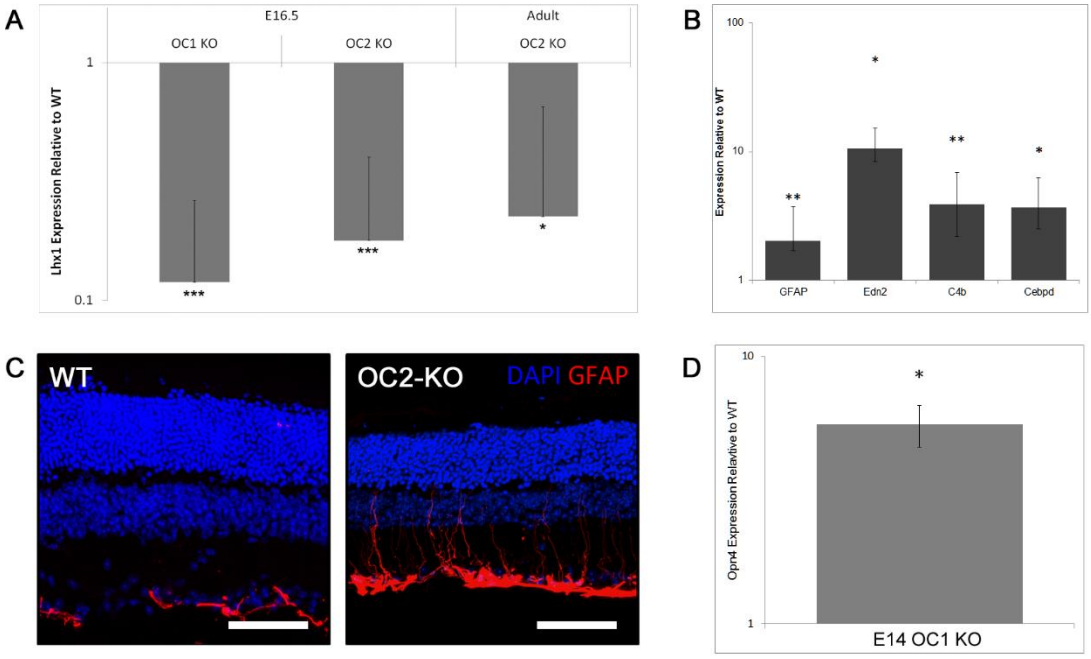
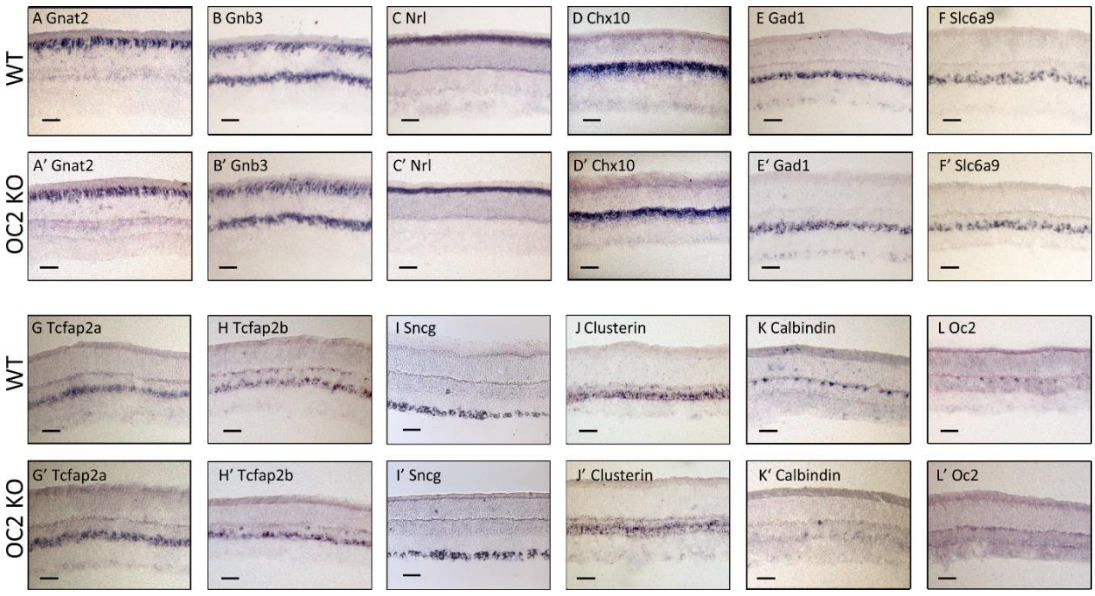


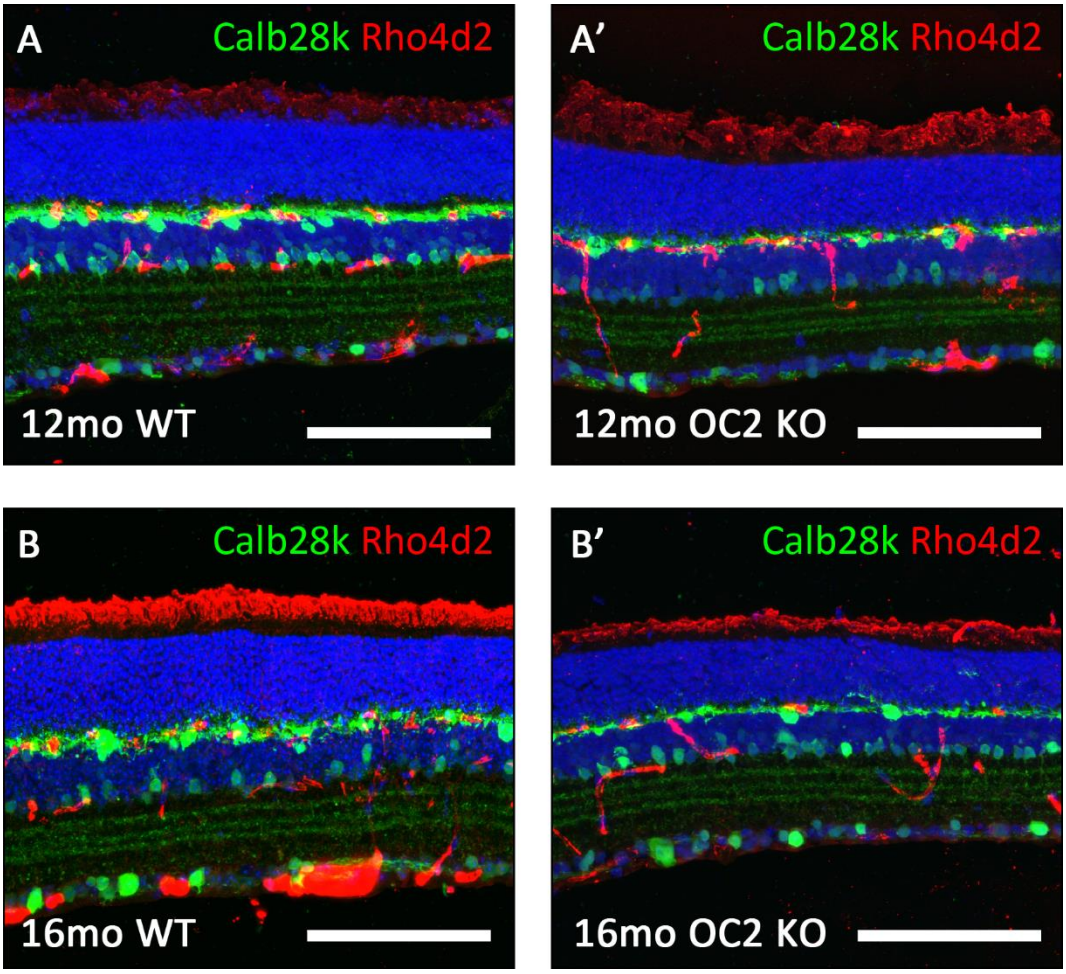
Figure 6. Unbiased search for changes in the OC2-deficient retina

(A) Quantitative PCR (qPCR) analyses determining the relative amounts of Lhx1 in OC1-KO, and OC2-KO retinas at E16.5 or adult compared to WT littermate retinas. (B) qPCR determining the relative amounts of various genes identified in models of retinal damage or disease expressed in adult OC2 retinas. Each marker known to be involved in retinal stress is significantly upregulated in OC2 compared to their WT littermates, including GFAP, Edn2, C4b, and Cebpd. (C) WT and OC2-KO retinas were stained with an anti-GFAP antibody. Scale bars represent 100 μm. (D) qPCR determining the relative amounts of Opn4 in E14.5 OC1-KO retinas compared to WT littermate retinas. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.005$. Scale bars represent 100 μm. All quantitative results are plotted on logarithmic scale.



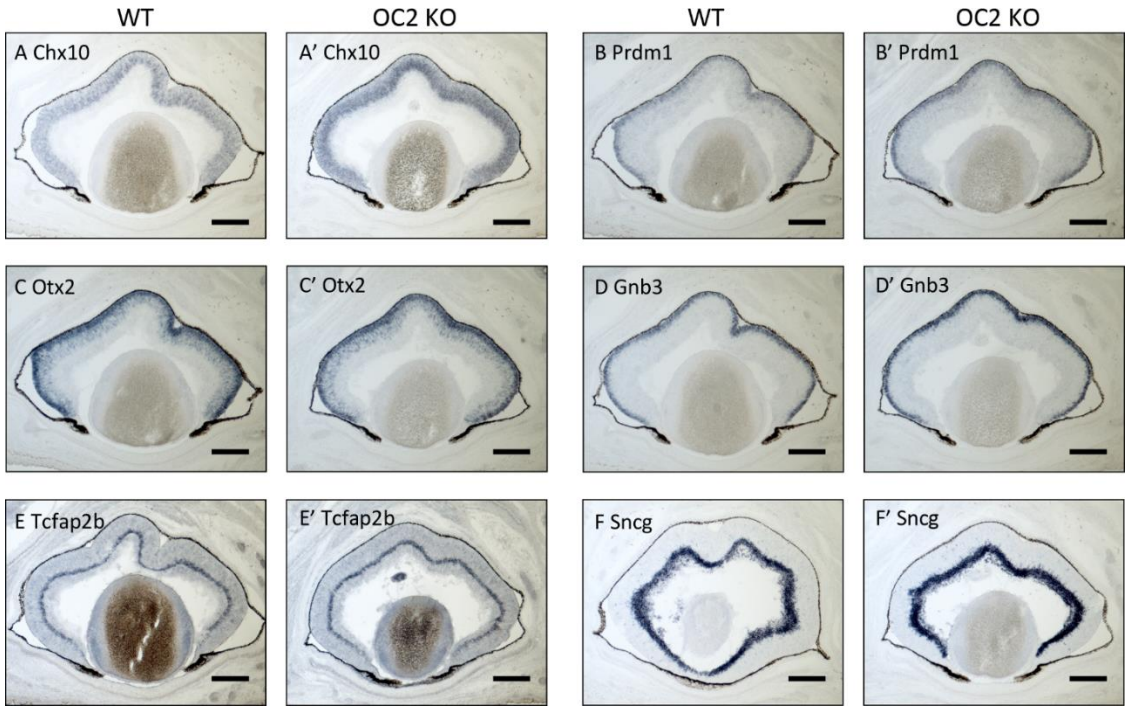
Supplemental Figure 1. Expression of adult retinal markers in OC2-KO retinas

In situ hybridization was employed to determine the effects of OC2-deficiency on adult retinal cells. Photoreceptors (A,A',B,B',C,C'), bipolar cells (D,D'), amacrine interneurons (E,E',F,F',G,G'.H,H'), ganglion cells (I,I') and Muller glia (J,J') appear unaffected by loss of OC2. However, the population of horizontal cells is greatly decreased in the absence of OC2 (K,K'). OC2 mRNA deficiency is seen in (L,L'). Scale bars represent 50 μm.



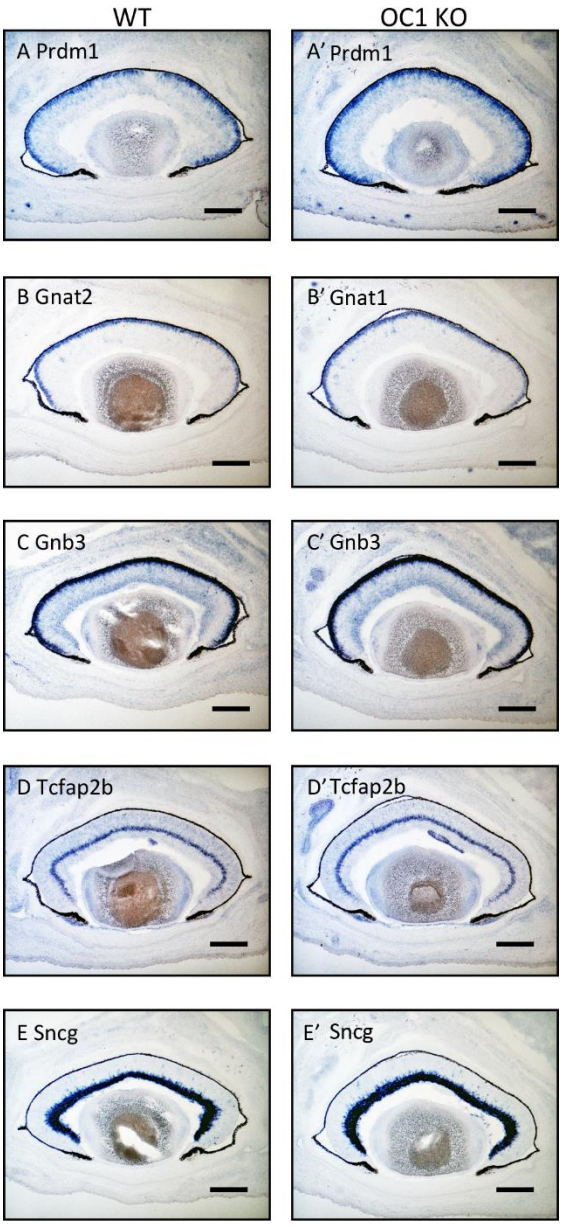
Supplemental Figure 2. Retinal degeneration in OC2-KO retinas from aged mice.

Immunohistochemistry was performed on WT and OC2 KO retinas from older mice to assess the integrity of the retinas. WT (A,C) and OC2-KO (B,D) littermates were stained with an anti-Rhodopsin antibody (Rho4d2) in red at 12 months of age (A,B) and 16 months of age (C,D). Anti-Calbindin 28k (Calb28k) staining is shown in green to illustrate the loss of horizontal cells in these OC2-KO retinas. Scale bars represent 100μm.



Supplemental Figure 3. Expression of retinal markers in developing E16.5 OC2-KO retinas

In situ hybridization was utilized to examine retinal progenitor cells (A,A'), developing photoreceptors (B,B',C,C',D,D'), amacrine cells (E,E'), and ganglion cells (F,F') in WT and OC2-deficient mouse retinas at E16.5. Scale bars represent 200 μm.



Supplemental Figure 4. Expression of retinal markers in developing E16.5 OC1-KO retinas

In situ hybridization was utilized to examine developing photoreceptors (A,A',B,B',C,C'), amacrine cells (D,D'), and ganglion cells (E,E') in WT and OC1-deficient mouse retinas at E16.5. Scale bars represent 200 μm.

Supplemental Tables 1-4:

The gene expression values resulting from Affymetrix microarrays of isolated WT or KO retinas were analyzed using R and the Bioconductor suite. Cel files were background-corrected and normalized using Mas5 and log(2) transformed. Shown are all genes found to be significantly differentially-expressed, where the average values of either WT or KO expression must exceed 7 (to indicate overall expression in either subset of transcriptomic data).

CHAPTER 3. THE EXPRESSION OF POLO-LIKE KINASE 3 AND ITS ROLE IN RETINAL DEVELOPMENT

1. Abstract

During retinogenesis seven different cell types are generated in distinct yet overlapping timepoints from a population of retinal progenitor cells (RPCs). At any given time, RPCs are at various stages of development and differentiation. Therefore, any tissue-wide analyses run the risk of averaging out the intrinsic signals that drive a progenitor towards a specific fate. Previously, we have performed single cell transcriptome analyses of RPCs to identify candidate genes that may play roles in the generation of early-born retinal neurons. Polo-like kinase 3 (Plk3) was identified as one such candidate gene that is highly expressed in subsets of early retinal cells. Although previous studies outside of the retina have determined that Plk3 and its family members can play roles in cell cycle maintenance, our preliminary studies indicate the kinase plays either an expanded or completely different role in the retina. We have obtained a Plk3-KO mouse and investigated changes in the retina's morphology and transcriptome through immunohistochemistry, in situ hybridization and gene expression profiling. These experiments have been performed initially on adult mice and subsequently extended throughout retinal development. Although morphological studies reveal few consistent changes in retinogenesis upon Plk3 loss, microarrays have revealed potential candidate genes changed in Plk3-KO mice. Further studies elucidating the

role of Plk3 in retinogenesis will be necessary to understand the difference in these phenotypes.

2. Introduction

Neural progenitor cells acquire their cell fates at prescribed times and places to ensure the right connections are formed in concert to generate an oftentimes intricately functioning and responsive tissue. Although numerous environmental and cell-intrinsic factors have been shown to contribute to the fate decisions of neural progenitor cells, there are many players still to be identified (Goetz et al., 2014a). Importantly, while single-cell transcriptomic analysis has shown that undifferentiated progenitors exhibit extreme diversity in their expression of known transcription factors and markers of various cell processes as well as many genes not yet associated with retinal functions (Trimarchi et al., 2008), the repercussions of this variation are not well understood. A better understanding of the effects of specific cell-intrinsic drivers, such as the genes that define heterogeneous subsets of progenitor cells, and their roles in the fate decisions that can vary even among a single cell's neighboring daughter cells, will greatly inform how a complex neural tissue is generated.

Central nervous system development can be modeled using the vertebrate retina due to its relative simplicity, laminar organization, and ease of isolation. The retina's six major neural types are organized into three nuclear layers

with rod and cone photoreceptors in the outer nuclear layer (ONL); horizontal, bipolar, and amacrine interneurons in the inner nuclear layer (INL); and retinal ganglion cells (RGCs) and displaced amacrine cells in the ganglion cell layer (GCL) (Masland, 2001). Together with Muller glia (whose nuclei can also be found in the INL), each retinal cell type is generated in a stereotyped timeline from a common pool of progenitors (Livesey and Cepko, 2001; Turner and Cepko, 1987; Turner et al., 1990). This developmental timeline is well-characterized and common to all vertebrates, beginning with ganglion cells and other early-born cell types such as cone photoreceptors, horizontal cells, and amacrine cells (Sidman, 1961; Young, 1985a, 1985b). Middle to late retinogenesis is characterized by the birth of rod photoreceptors, bipolar cells, and Muller glia (Cepko, 1996; Sidman, 1961; Young, 1985a, 1985b). Clonal analyses have determined that retinal progenitors are multipotent, or capable of producing more than one type of retinal cell throughout development (Turner and Cepko, 1987; Turner et al., 1990). Additionally, these progenitor cells have been shown to exhibit considerable gene expression heterogeneity throughout the different stages of retinogenesis (Trimarchi et al., 2008). While the exact mechanism by which dividing retinal progenitors assume a cell fate is as yet unknown, studies of individual retinal progenitors have served to clarify some facets of the variable transcriptomes displayed by developing cells, including the discovery of novel markers and temporal patterns in the generation of neural subsets (Trimarchi et al., 2008).

Math5 is a bHLH transcription factor expressed in retinal progenitor cells during the G2/M phase of the cell cycle when cell fates are most likely being

acquired (Brown et al., 1998; McConnell and Kaznowski, 1991). In the mouse, a subset of early-generated neural progenitors, including photoreceptors, amacrine cells, horizontal cells, and a majority of ganglion cells show a history of Math5 expression (Brown et al., 1998; Feng et al., 2010; Skowronska-Krawczyk et al., 2009; Yang et al., 2003). Furthermore, Math5 and its homologues are necessary for ganglion cell generation and optic nerve formation in multiple vertebrates, including zebrafish and mice (Brown et al., 2001; Kay et al., 2001; Wang, 2001). Misexpression and knockdown of Math5 has been shown to lead to altered proportions of other early retinal cells, indicating that this transcription factor is important in early retinal cell fate determination (Brown et al., 2001; Feng et al., 2010; Kay et al., 2001; Liu et al., 2001). Given the critical and conserved expression of Math5 in early retinogenesis, we identified genes highly correlated with Math5 expression in the transcriptomes of single retinal progenitors and developing neurons isolated throughout retinogenesis (Trimarchi & Cepko, *in preparation*). Among the genes most highly correlated with Math5 expression in single retinal cells was Polo-like kinase 3 (Plk3). Further investigation of Plk3's expression within the single-cell dataset led to the discovery that in addition to Math5, Plk3 correlated strongly with Dlx1 and Dlx2, which are also known to be important for the terminal differentiation of late-born ganglion cell populations (de Melo et al., 2005).

The vertebrate polo-like kinases, much like their *Drosophila* homolog, Polo, are key cell cycle regulators (Zimmerman and Erikson, 2007a). Previous work has indicated that while Plk3 is detectable throughout the cell cycle, peak Plk3 protein expression occurs during the G1 phase of the cell cycle in cultured human cells.

Further experimentation demonstrated that Plk3 is a key regulator of the G1 to S phase transition through post-transcriptional attenuation of CyclinE, possibly in conjunction with its substrate, Cdc25A (Iida et al., 2009; Myer et al., 2005; Zimmerman and Erikson, 2007b), indicating a potential role in directing the cell cycle of retinal progenitors. Additionally, Plk3 has been linked to the p53 pathway, possibly playing a role in cell cycle arrest and apoptosis (Xie et al., 2001). Other studies have implicated Plk-family members in processes separated from the cell cycle. For example, Plk2 and Plk3 have been linked to the integrity of hippocampal neurites and synaptic plasticity, indicating a possible role in the maturation of retinal neurons (Pak and Sheng, 2003; Seeburg et al., 2005). Plk3 has also been shown to phosphorylate alpha- and beta-synucleins and along with its family members is often co-localized with phosphorylated synucleins (Mbefo et al., 2010).

With the knowledge that Plk3 is coexpressed with Math5 in developing retinal progenitors, as well the possible array of responsibilities for this kinase and its family members in differentiating and maturing neurons, we have obtained a Plk3-knockout (KO) mouse (Myer et al., 2011) and characterized its retinal phenotype to understand the role of Plk3 in the developing retina. We have confirmed that Plk3 is expressed in differentiating retinal cells throughout the early stages of retinogenesis. However, extensive morphological analyses of mature and developing Plk3-KO mice have determined that loss of Plk3 does not lead to noticeable disruptions in normal retinal development. Although abnormalities were in the organization of the inner plexiform layer of some Plk3 deficient mice, this effect was not reproducible. Transcriptomic analysis using microarrays have shown

significant changes in the expression levels of multiple gene markers that were confirmed by qPCR, but the downstream effects of these transcriptomic changes were not noted in morphological analysis. Although we did not observe upregulation of other Plk family members at the RNA level, we cannot rule out some form of compensatory mechanism occurring during development in Plk3-KO mice.

3. Materials and methods

Ethics statement

All procedures for the care and housing of mice conform to the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Genotyping

Plk3 deficient mice were obtained (Peter Stambrook, University of Cincinnati College of Medicine) and genotyped as described (Myer et al., 2011). Plk3 knockouts were defined by the presence of the KO band (F: 5'-AAACCACCTGTGTTGGTGATGTGC-3'; R: 5'-AGCTAGCTTGGCTGGACGTAAACT-3')

whereas wildtype littermates were identified by the presence of a WT band (F: 5'-TTTCCTGGAGCTCTGTAGCCGAAA-3'; R: ACACCCATCTGTGCCATACACTCA-3'). All four primers were used in the same reaction according to the same program: 3 min at 95°C; then 37 repetitions of 30s at 95°C, 10s at 60°C, 1 min at 72°C; followed by 7 min at 72°C. The products were separated on a 2% gel.

Tissue processing for cryosectioning

Immediately upon euthanasia, eyes were placed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight (O/N) at 4°C. After three 15 minute washes in PBS, retinas were isolated and rocked in a solution of 30% sucrose in PBS until they sunk (time dependent on mouse age). OCT solution (Tissue-Tek) was added to a concentration of 50% OCT/ 50% sucrose in PBS and retinas were rocked until the solution equilibrated. Upon equilibration, retinas were frozen at -80°C until cryosectioning. Retinas were sectioned at 20um and sections placed onto Superfrost Plus microscope slides (Fisher Scientific).

***In situ* hybridization**

Sequences (between 650 and 800bp in length) were amplified by PCR from isolated mouse cDNA (see supplemental table for details). Probes were visualized using an α-DIG-AP antibody (Roche) and subsequent exposure using BCIP and NBT (Trimarchi, et al., 2007; Goetz et al 2014b). The 3'-targeted Plk3 probe used in this

study was from the BMAP collection, accession number AW488956, the sequence for which was confirmed before use. A probe was also designed for the center of the gene (L: tgtctcctgcttggtgagtg; R: cccgtagaagttcacctgga) and the 5' portion (L: ctcatcaccgaccctctcag, R: ttgatgcagcggtatgtctc). Fluorescent dissociated cell in situ hybridization was also performed as described (Trimarchi et al., 2007). Briefly, one probe was synthesized as digoxigenin (DIG)-labeled and the other probe was labeled with fluorescein. Tyramide amplification (Life Technologies) was performed for 10 minutes for the first probe, followed by inactivation in 0.3% hydrogen peroxide. The second color was then processed in exactly the same manner as the first probe, but with a second color. Six independent fields were photographed and quantified.

Antibody stains

Slides were blocked for 30m in PBS-Triton (1% BSA, 0.01% Triton X-100, 0.004% SDS) and then placed in primary antibody, diluted according to manufacturer's instructions in PBS-Triton, O/N at 4°C. Slides were washed in PBS-Triton three times for 15 min. at room temperature (RT). Secondary antibodies were diluted according to manufacturer's instructions in PBS-Triton and applied either for 2-4h at RT or O/N at 4°C. After this incubation, slides were again washed three times for 15 min. at RT, and mounted with Fluoromount-G (Southern Biotech).

Primary antibodies used were anti-Calbindin28K (1:2000; Swant, Switzerland), anti-Calretinin (1:1000; Millipore), anti-Chx10 (1:1000; Morrow et al.,

2008), anti-Glutamine Synthetase (1:10,000; Sigma), anti-Rhodopsin (1:100; Molday and MacKenzie, 1983), anti-Chat (1:100; Millipore), Anti-PKC (1:10,000; Sigma-Aldrich), anti-Recoverin (1:100; courtesy Don Sakaguchi), anti-Brn3b (1:100; Santa Cruz Biotechnology), and anti-PH3 (1:500; Millipore). The anti-Pax6 (1:50) and anti-Islet1 (1:50) antibodies were obtained from the Developmental Studies Hybridoma Bank (DHSB), developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City.

Microarrays

Microarray hybridization was performed as described previously (Goetz et al., 2014b). Briefly, RNA was isolated from retinas using Tri-reagent (Sigma) according to standard manufacturer's protocols. 400ng of total RNA was used to generate aRNA, from which 5ug were fragmented using the Ambion MessageAmp™ II aRNA Amplification Kit according to manufacturer's instructions. Samples were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays at the GeneChip facility at Iowa State University. Microarray results were analyzed using the Bioconductor Affy package in R. Mas5 was employed for background adjustment and normalization and all data were log(2) transformed. We limited the differential expression analyses to only those genes whose mean log(2) transformed expression value in either WT or KO retinas exceeded the minimum cutoff of 7 as this level of expression was consistently labeled as present by the Affymetrix algorithm. A two-tailed t-test which resulted in p-values of less than 0.05 indicated significant differential expression.

qPCR

Retinal RNA was isolated using Tri-reagent (Sigma) according to manufacturer's instructions. 400ng of RNA was used to generate cDNA using random primers and SuperScript III (Life Technologies) according to standard protocols. SybrGreen MasterMix (ThermoFisher) was used to perform qPCR in a BioRad CFX96 Real Time System with BioRad C1000 Thermal Cycler. The program used for qPCR was: 15m at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 56°C, and 30 sec at 72°C. β-Actin was used to normalize each experimental gene, and further analysis was performed as described (Livak and Schmittgen, 2001). The difference in average ΔΔC(t) values and the difference plus and minus the standard error of the difference were computed on the C(t) scale. The base-2 antilogs of these three values were computed to obtain estimates with error bars on the fold change scale. Results were plotted on a logarithmic scale. qPCR primers were designed as follows: Plk3-3' (f:cctgcttaggttccaactcg; r:taaagctggtccctgattcc), Plk3-5' (f:ggtatagcctacgcggtcaa; r:tgtcagcatcctcgaaatga), Rp1 (f:cctatgtccactccctccaa; r:ccagcctggaaaccatacat), Tac1 (f:gatgaaggagctgtccaagc; r:cagcatgaaagcagaaccag).

4. Results

Characterization of Plk3 expression in the mouse retina

To better understand the mechanisms of cell fate determination in the retina, transcriptomes of single retinal progenitors were analyzed. Given that Math5 is a marker of the transition from retinal progenitor cell to differentiating neuron, we focused on genes that correlated strongly with Math5 (Trimarchi & Cepko, in preparation). Among the genes preferentially expressed in Math5+ retinal progenitors was Plk3 (Figure 1A). In fact, Math5 and Plk3 were among the highest correlated genes in a dataset of embryonic single cells with heterogeneous gene expression (Trimarchi et al., 2008, and *in preparation*). The polo-like family of kinases are well-characterized regulators of cell division in mammalian cells (Iida et al., 2009; Zimmerman and Erikson, 2007a, 2007b). Given the onset of Math5 expression in G2/M progenitors and the fact that the polo-like kinases may behave redundantly (Strebhardt, 2010), we examined the expression of other Polo-like kinases in our transcriptome dataset of embryonic single cells. It was interesting to note that while other family members, including Plk1 and Plk4, were expressed in subsets of our progenitor cell transcriptomes, neither Plk3 nor Math5 clustered with other polo-like kinases (Figure 1A). To determine whether Plk3 expression in the retina correlated with other markers of cell cycle progression, we looked at the expression of G2/M marker genes in Plk3+ and Plk3- cells (Figure 1B). Despite

Plk3's previously described functionality as a cell cycle regulator and the role of these kinases in G2/M progression (Wang et al., 2002; Zimmerman and Erikson, 2007b), there was no correlation between Plk3 expression and other markers of G2/M during retinogenesis. In fact, Plk3+ cells were less likely to exhibit markers of G2/M than the Plk3- cells in our set of embryonic retinal progenitor cells.

We sought to further characterize the expression of Plk3 throughout the course of retinogenesis. In situ hybridization was performed on frozen retinal sections derived from developing mice. Plk3 mRNA was detected most strongly in the inner neuroblast layer of the central retina at E12, spread to the periphery by E14, and eventually decreased to undetectable levels in all but the peripheral retina by E16 (Figure 1C). Interestingly, the location of Plk3 expression at E14 overlaps with developing amacrine cells (Figure 1C). Other markers of maturing amacrine interneurons (Figure 1D) have notably similar patterns of expression at this stage in neurogenesis. These expression patterns place Plk3 at a time and place to affect the normal development of early-generated retinal cells. Since Math5 and Plk3 were co-expressed in individual retinal cells on microarrays, we wished to explore the specific overlap between larger numbers of Math5+ and Plk3+ cells by labeling them in tandem through dissociated fluorescent *in situ* hybridization (Figure 1E). In dissociated E14 retinal cells, 10.2% of cells expressed Plk3, while 13.4% of cells expressed Math5, and 2.8% of all cells coexpressed probes for both Plk3 and Math5 (Figure 1F).

Characterization of mature and postnatal Plk3 deficient retinas

Since we observed that Plk3 expression in the early retina was correlated with Math5 expression and with the production of early-generated retinal neurons, we decided to investigate the function of Plk3 in the developing retina. A Plk3-KO mouse has been previously generated to study the kinase's role in tumorigenesis, but the retinal development of these mice had not been characterized (Myer et al., 2011). We obtained the mice (Myer et al., 2011) and upon initial characterization noticed that their retinas were degenerated. This was because the mice were partially in a Black Swiss background, which carried a fast-acting allele of retinal degeneration ($Pde6b^{-/-}$, Rd1). To remove the Rd1 allele from the line, mice were crossed with C57Bl/6 mice until all litters were wildtype at the Rd1 locus as assessed by genotyping PCR.

To determine whether Plk3 loss led to gross disturbances in retinal morphology, we performed antibody stains on adult Plk3-KO retinas and their wildtype (WT) littermates (Figure 2). These surveys of retinal cell populations showed no qualitative differences in populations of rod photoreceptors (Rho4d2, Figure 2A,A'), bipolar cells (α-Chx10, Figure 2B,B'; α-PKC-gamma, Figure 2C,2C'), Muller glia (α-glutamine synthetase, Figure 2D,D'), amacrine cells (α-Isl1, α-Pax6, α-Chat; Figure 2E-G,E'-G'), retinal ganglion cells (α-Brn3b, Figure 2H,H'), or horizontal cells (α-Calb28k, α-Calretinin; Figure 2I,I') between Plk3-KO and WT mice. Notably,

while IPL staining sometimes appeared disorganized in Plk3-KO retinal sections (Figure 2G,G'this staining pattern was not consistently reproducible in all Plk3-KO mice. To quantify this finding, WT and Plk3-deficient retinas from three independent litters were stained with an anti-Calb28k antibody, randomized, and blindly scored on quality of IPL stratification by a graduate student familiar with retinal morphology. The scores, given on a scale of 1 (highly disorganized) to 5 (highly stratified) did not display significant differences in IPL quality (n=7 WT, n=9 KO; p=0.12). To further investigate the possible presence of an IPL phenotype under different tissue processing and sectioning conditions, antibody stains were also performed on retinal sections processed using paraffin-wax. While slight disorganization was observed in the IPL of Plk3-KO mice (n=1, Figure S1), the changes were not so dramatic as to confirm a phenotype alone.

To identify whether transient phenotypes existed during retinal development in the Plk3-KO mouse, immunohistochemistry was performed on retinas at an early stage [Postnatal Day (P)7] to determine whether changes to retinal subpopulations were apparent upon the establishment of retinal connectivity. Antibody stains at P7 indicated no observable changes between populations of photoreceptors (α-Rho4d2, Figure 3A,A'), bipolar cells (α-Chx10, Figure 3B,B'), ganglion cells (α-Brn3b, Figure 3C,C'), amacrine cells (α-Pax6, Isl1; Figure 3D-E,D'-E'), and the combination of horizontal cells and amacrine cells (α-Calbindin28k, α-Calretinin; Figure 3F.) Again, at this time point, multiple replicates were used to determine whether possible IPL phenotype was present by this earlier point in retinal maturation; however, the phenotype was not reproducible (n=3 WT

and KO pairs). To further confirm the lack of gross phenotypes in the Plk3-KO retina, antibody stains were also performed at an earlier (P4, Figure S3) and later (P14, Figure S4) time point. At P4, again, no phenotype was discovered in populations of maturing photoreceptors (α-Recoverin, Figure S3A,A'), bipolar cells (α-Chx10, Figure 3B,B'), ganglions (α-Brn3b, Figure 3C,C'), amacrine interneurons (α-Pax6, Isl1; Figure 3D-E,D'-E'), or horizontal cells and amacrines (α-Calbindin28k, α-Calretinin; Figure 3F). Similarly, no changes were found in the same populations of cells at P14 (Figure S4).

Unbiased transcriptomic screening of Plk3 knockout retinas

To better understand the role that Plk3 may play in retinal development, we looked for the presence of more subtle changes in adult retinas that were either wildtype or null for Plk3. Full transcriptomic analysis of murine retinas not only reveal phenotypes that are not immediately apparent upon gross morphological analysis, but also provides an unbiased determinant of changes in even small neural subpopulations (Goetz and Trimarchi, 2015; Goetz et al., 2014b). Therefore, microarray analysis was performed on adult Plk3-KO retinas and their littermates (n=3 Plk3-KO, n=3 WT). Surprisingly, among the genes with the highest increase in microarray-based expression in our Plk3-KO mice was consistently Plk3 itself. As in previous studies with transgenic knockout mice (Goetz et al., 2014a), the microarray probe was confirmed to target the 3' end of the gene, beyond the extent of the knocked-out region. A qPCR probe designed to the same portion of the gene confirmed significant upregulation ($p<0.01$)(Figure 4B). However, a qPCR probe

targeting the functional domain of Plk3 showed extreme downregulation, further confirming the fact that the Plk3 coding region had been removed ($p<0.05$)(Figure 4B). Additionally, to establish that the observed expression was indicative of the entire length of the coding sequence of Plk3, ISH probes to two additional regions of Plk3's coding sequence were designed. ISH analysis from all three probes confirmed that the expression patterns of each probe spanning the length of Plk3 closely mirrored Math5 expression in WT retinas, as well as the lack of Plk3 expression in our Plk3-KO retinas (data not shown).

We next searched for additional significantly altered genes in Plk3-KO retinas when compared to wildtype littermates and found a few markers of photoreceptors and ganglion cells were upregulated, while markers of Muller glia, bipolar cells, and amacrine markers were decreased in comparison to WT littermates. Downregulation of Tac1, a marker of subsets of amacrines, was confirmed using real-time quantitative PCR (qPCR)($p<0.05$), whereas retinitis pigmentosa 1 (Rp1), a marker of photoreceptors, was significantly upregulated ($p<0.05$) (Figure 4A).

In situ hybridization was performed to determine whether Plk3 deficiency resulted in defects in smaller subsets of mature retinal cells (Figure S2). Of particular interest to us were subsets of cones marked by Opn1sw and Rp1l1 (Figure S2A,A',B,B') and subsets of amacrine cells (Figure S2C-J,C'-J'). Bipolar cells were also visualized using a probe against Sebox (Figure S2K,K'), and Muller glia

were marked with a probe for Vimentin (Figure S2L,L'). However, no distinctive differences were observed between WT and Plk3-KO littermates.

We also wanted to compare the significantly differentially expressed genes from the adult microarrays to the genes that may be changed during development. Therefore, we isolated retinas from P7 littermates and performed microarray analysis to determine genes with differential expression in retinas that were in the process of generating intercellular connectivity. At P7, among the most highly upregulated genes in Plk3-KO retinas was the cone-specific gene Pde6h. We also performed microarray analysis on P0 retinas to determine earlier changes in retinal transcriptomes, to find that the cell cycle marker Cdc20 was significantly upregulated in Plk3-deficient retinas.

Overall, multiple genes were consistently changed between the nine samples studied in all three time points, including the aforementioned Plk3 microarray probe, which was among the most upregulated genes in the retina (n=9 WT, 9KO) confirming that transcriptome preparation was highly reproducible. Genes related to RNA binding and transcription such as Khdrbs1 were upregulated in the absence of Plk3, as well as Med8 which has high homology to markers of GABAergic neurons in *C. elegans,* although its role in mammalian neurons has not been fully explored (Hammock et al., 2010). Other genes showing significant differential expression include those with as-yet undescribed function in the retina or as direct effectors of Plk3, including Med8 which was highly downregulated in Plk3-KO retinas. Additionally, Cap1, which played a noted role in promoting actin cytoskeleton

dynamics (Bertling, 2004), was highly and consistently upregulated among all postnatal timepoints studied. A GO enrichment survey was performed using DAVID to determine whether there were any trends in the genes which were significantly differentially expressed at any of the studied timepoints. GO analysis showed that genes that were significantly upregulated in Plk3-KO retinas were significantly enriched for terms including phosphorylation and kinase-related genes, positive regulation of transcription and RNA processing, cell differentiation and nuclear export. Enriched GO terms for genes downregulated in the absence of Plk3 included ATP-binding, transcriptional regulation, and cell projection or neurite-related genes.

Effects of Plk3 loss during embryonic stages of retinogenesis

The expression patterns of Plk3 during midembryonic retinal development warranted further investigation into the role being played by Plk3 during this critical period in cell proliferation and fate determination. Therefore, we performed a full survey of the cell types and transcriptome of E16 embryonic retinas to conclusively determine whether or not Plk3 loss would lead to even minute or transient changes in retinogenesis. First, *in situ* hybridization was performed to compare the expression of Plk3 and its family members during Plk3's normal period of expression, E14 (Figure S5). While confirming the loss of Plk3 at the mRNA level (Figure S5A,A'), embryonic *in situs* also established that while other Plk family members are present at comparatively lower stages at this stage of retinal development, they do not appear to change expression levels upon Plk3 loss in any compensatory fashion.

Plk3 experiences a peak of expression around mid-embryogenesis and then begins to decrease by E16, by which time the effects of its loss may be apparent. *In situ* hybridization was performed on Plk3-KO mice and their littermates to determine changes in progenitor populations (Chx10, Figure S6A, A'), retinal ganglion cells (Syng, Figure S6B,B'; Brn3b, C,C'; Ebf3, D,D'), amacrine interneurons (Ap2a, Figure S6E,E'; Ap2b, F,F'), and developing photoreceptors (Rxrg, Figure S6G, G'; Otx2, H,H'). No discernable differences were noted in these developing populations.

To better understand the changes that may result from Plk3 loss in the mid-embryonic retina, including those that would not be apparent from morphological surveys, transcriptomic analysis of E16 retinas was performed. To ensure the lowest variation, retinas from 3 Plk3-KO and WT littermates from the same litter were isolated. GO analysis determined that clusters of genes associated with catabolic processes, cell and neurite projection, and phosphorylation were significantly enriched in WT Plk3 mice compared to their Plk3-null littermates at E16. Conversely, genes significantly overexpressed in Plk3-KO mice disproportionally represented clusters of genes associated with ion binding, chromatin organization and regulation, RNA binding and transcriptional processes. Of particular interest among differentially-expressed genes was the retinitis pigmentosa GTPase regulator interacting protein (Rgrip1), which was highly upregulated in the absence of Plk3. Axon targeting molecules and receptors such as Sema3f, Sema4d, Epha8, and Ephb3 were downregulated to varying degrees, but as these changes led to no noticeable

downstream defects in dendritic morphology in adult retinas. The repercussions of these decreases remain unknown.

5. Discussion

Plk3 is a member of the polo-like family of kinases, known to play roles in cell cycle maintenance (Zimmerman and Erikson, 2007a). In particular, Plk3 has been hypothesized to work together with Cdc25a to decrease CyclinE levels posttranscriptionally as a means of regulating progression from G1 to S phase of the cell cycle (Iida et al., 2009; Myer et al., 2005; Zimmerman and Erikson, 2007b). Plk3 and its family member Plk2 have also been implicated in synaptogenesis and maintenance of neurite integrity (Pak and Sheng, 2003; Seeburg et al., 2005). Despite its well-described roles in multiple important cellular processes, Plk3 has not yet been described in the retina, so we were especially intrigued to identify Plk3 among the genes with highest correlation to Math5 expression in the developing retina (Figure 1A). Surveys of Plk3's expression throughout neurogenesis indicated that this gene was expressed in patterns reminiscent of Math5 expression, with strong expression following a wave of neurogenesis in the midembryonic retina (Figure 1C). This expression pattern was also reminiscent of markers of developing amacrine cells such as Tcfap2b, Tcfap2d, and Nhlh2 at E14 (Figure 1D). Studies of knockouts for members of each of these two families of transcription factors showed no discernable phenotype (Kruger and Braun, 2002) or subtle phenotypes, possibly resulting from functional redundancy among family members (Bassett et al., 2012).

To better understand the role played by Plk3 in the neural retina, we obtained a Plk3-KO mouse (Myer et al., 2011) and performed a thorough morphological and transcriptomic survey of the differences present between Plk3-KO mice and their WT littermates throughout development. No distinctive morphological phenotypes were readily reproducible in developing or mature Plk3-KO mice, and few transcriptomic changes could be readily confirmed by other methods such as qPCR or *in situ* hybridization.

The Plk family members exhibit high levels of conservation that could indicate overlapping functionality (Strebhardt, 2010). While we noted no significant changes in the mRNA expression levels of other Plk family members upon loss of Plk3 (Figure S5), their expression in the retina does not preclude that even small amounts of enzymatic activity from other family members could compensate to prevent a Plk3-null phenotype.

There have been multiple examples of nonphenotypic mice that exhibit genetic robustness; that is, the knocked-out gene carries no observable phenotypic weight as a result of adaptation or as an inherent feature of a genetic pathway with built-in failsafe mechanisms (Barbaric et al., 2007). An additional concern when considering the phenotype of a knockout mouse is the variability in background strain and mechanism of generation of the knockout, including the nature of deleted exons. For instance, while the mouse used in this study was generated with a knockout in the promoter region as well as exons 1-6, was not shown to contribute to tumorigenesis (Myer et al., 2011). However, another Plk3-KO mouse generated by

removal of exons 1-8 did show increased tumor formation in mice of advanced age (Yang et al., 2008). Either excerpt from the Plk3 gene included the active sites for the gene. However, the possibility remains that the background of mice or the methods employed to identify a phenotype contributed to either study's findings as well as those of the current investigation.

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7. References

Barbaric, I., Miller, G., and Dear, T.N. (2007). Appearances can be deceiving: phenotypes of knockout mice. Brief. Funct. Genomic. Proteomic. *6*, 91–103.

Bassett, E.A., Korol, A., Deschamps, P.A., Buettner, R., Wallace, V.A., Williams, T., and West-Mays, J.A. (2012). Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. Dev. Dyn. *241*, 814–829.

Bertling, E. (2004). Cyclase-associated Protein 1 (CAP1) Promotes Cofilin-induced Actin Dynamics in Mammalian Nonmuscle Cells. Mol. Biol. Cell *15*, 2324–2334.

Brown, N.L., Kanekar, S., Vetter, M.L., Tucker, P.K., Gemza, D.L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Dev. Camb. Engl. *125*, 4821–4833.

Brown, N.L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Dev. Camb. Engl. *128*, 2497–2508.

Cepko, C.L. (1996). The patterning and onset of opsin expression in vertebrate retinae. Curr. Opin. Neurobiol. *6*, 542–546.

Feng, L., Xie, Z., Ding, Q., Xie, X., Libby, R.T., and Gan, L. (2010). MATH5 controls the acquisition of multiple retinal cell fates. Mol. Brain *3*, 36.

Goetz, J.J., and Trimarchi, J.M. (2015). Transcriptomic analyses of Onecut1 and Onecut2 deficient retinas. Genomics Data *4*, 88–89.

Goetz, J.J., Farris, C., Chowdhury, R., and Trimarchi, J.M. (2014a). Making of a Retinal Cell. In International Review of Cell and Molecular Biology, (Elsevier), pp. 273–321.

Goetz, J.J., Martin, G.M., Chowdhury, R., and Trimarchi, J.M. (2014b). Onecut1 and Onecut2 Play Critical Roles in the Development of the Mouse Retina. PLoS ONE *9*, e110194.

Hammock, E.A., Eagleson, K.L., Barlow, S., Earls, L.R., Miller, D.M., and Levitt, P. (2010). Homologs of genes expressed in Caenorhabditis elegans GABAergic neurons are also found in the developing mouse forebrain. Neural Develop. *5*, 32.

Iida, M., Sasaki, T., and Komatani, H. (2009). Overexpression of Plk3 causes Morphological Change and Cell Growth Suppression in Ras Pathway-activated Cells. J. Biochem. (Tokyo) *146*, 501–507.

Kay, J.N., Finger-Baier, K.C., Roeser, T., Staub, W., and Baier, H. (2001). Retinal Ganglion Cell Genesis Requires lakritz, a Zebrafish atonal Homolog. Neuron *30*, 725–736.

Kruger, M., and Braun, T. (2002). The Neuronal Basic Helix-Loop-Helix Transcription Factor NSCL-1 Is Dispensable for Normal Neuronal Development. Mol. Cell. Biol. *22*, 792–800.

Liu, W., Mo, Z., and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. Proc. Natl. Acad. Sci. U. S. A. *98*, 1649–1654.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods *25*, 402–408.

Livesey, F.J., and Cepko, C.L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. *2*, 109–118.

Masland, R.H. (2001). The fundamental plan of the retina. Nat. Neurosci. *4*, 877–886.

Mbefo, M.K., Paleologou, K.E., Boucharaba, A., Oueslati, A., Schell, H., Fournier, M., Olschewski, D., Yin, G., Zweckstetter, M., Masliah, E., et al. (2010).

Phosphorylation of Synucleins by Members of the Polo-like Kinase Family. J. Biol. Chem. *285*, 2807–2822.

McConnell, S., and Kaznowski, C. (1991). Cell cycle dependence of laminar determination in developing neocortex. Science *254*, 282–285.

De Melo, J., Du, G., Fonseca, M., Gillespie, L.-A., Turk, W.J., Rubenstein, J.L.R., and Eisenstat, D.D. (2005). Dlx1 and Dlx2 function is necessary for terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina. Dev. Camb. Engl. *132*, 311–322.

Myer, D.L., Bahassi, E.M., and Stambrook, P.J. (2005). The Plk3-Cdc25 circuit. Oncogene *24*, 299–305.

Myer, D.L., Robbins, S.B., Yin, M., Boivin, G.P., Liu, Y., Greis, K.D., Bahassi, E.M., and Stambrook, P.J. (2011). Absence of polo-like kinase 3 in mice stabilizes Cdc25A after DNA damage but is not sufficient to produce tumors. Mutat. Res. *714*, 1–10.

Pak, D.T.S., and Sheng, M. (2003). Targeted protein degradation and synapse remodeling by an inducible protein kinase. Science *302*, 1368–1373.

Seeburg, D.P., Pak, D., and Sheng, M. (2005). Polo-like kinases in the nervous system. Oncogene *24*, 292–298.

Sidman, R. (1961). Histogenesis of mouse retina studied with thymidine-H3. In The Structure of the Eye, G. Smelser, ed. (New York: Academic Press), pp. 487–506.

Skowronska-Krawczyk, D., Chiodini, F., Ebeling, M., Alliod, C., Kundzewicz, A., Castro, D., Ballivet, M., Guillemot, F., Matter-Sadzinski, L., and Matter, J.-M. (2009). Conserved regulatory sequences in Atoh7 mediate non-conserved regulatory responses in retina ontogenesis. Development *136*, 3767–3777.

Strebhardt, K. (2010). Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. Nat. Rev. Drug Discov. *9*, 643–660.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual Retinal Progenitor Cells Display Extensive Heterogeneity of Gene Expression. PLoS ONE *3*, e1588.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Wang, S.W. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev. *15*, 24–29.

Wang, Q., Xie, S., Chen, J., Fukasawa, K., Naik, U., Traganos, F., Darzynkiewicz, Z., Jhanwar-Uniyal, M., and Dai, W. (2002). Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. Mol. Cell. Biol. *22*, 3450–3459.

Xie, S., Wu, H., Wang, Q., Cogswell, J.P., Husain, I., Conn, C., Stambrook, P., Jhanwar-Uniyal, M., and Dai, W. (2001). Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. J Biol Chem *276*, 43305–43312.

Yang, Z., Ding, K., Pan, L., Deng, M., and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. *264*, 240–254.

Young, R.W. (1985a). Cell proliferation during postnatal development of the retina in the mouse. Dev. Brain Res. *21*, 229–239.

Young, R.W. (1985b). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

Zimmerman, W.C., and Erikson, R.L. (2007a). Finding Plk3. Cell Cycle Georget. Tex *6*, 1314–1318.

Zimmerman, W.C., and Erikson, R.L. (2007b). Polo-like kinase 3 is required for entry into S phase. Proc. Natl. Acad. Sci. *104*, 1847–1852.

Zimmerman, W.C., and Erikson, R.L. (2007c). Polo-like kinase 3 is required for entry into S phase. Proc. Natl. Acad. Sci. *104*, 1847–1852.

8. Figures and legends

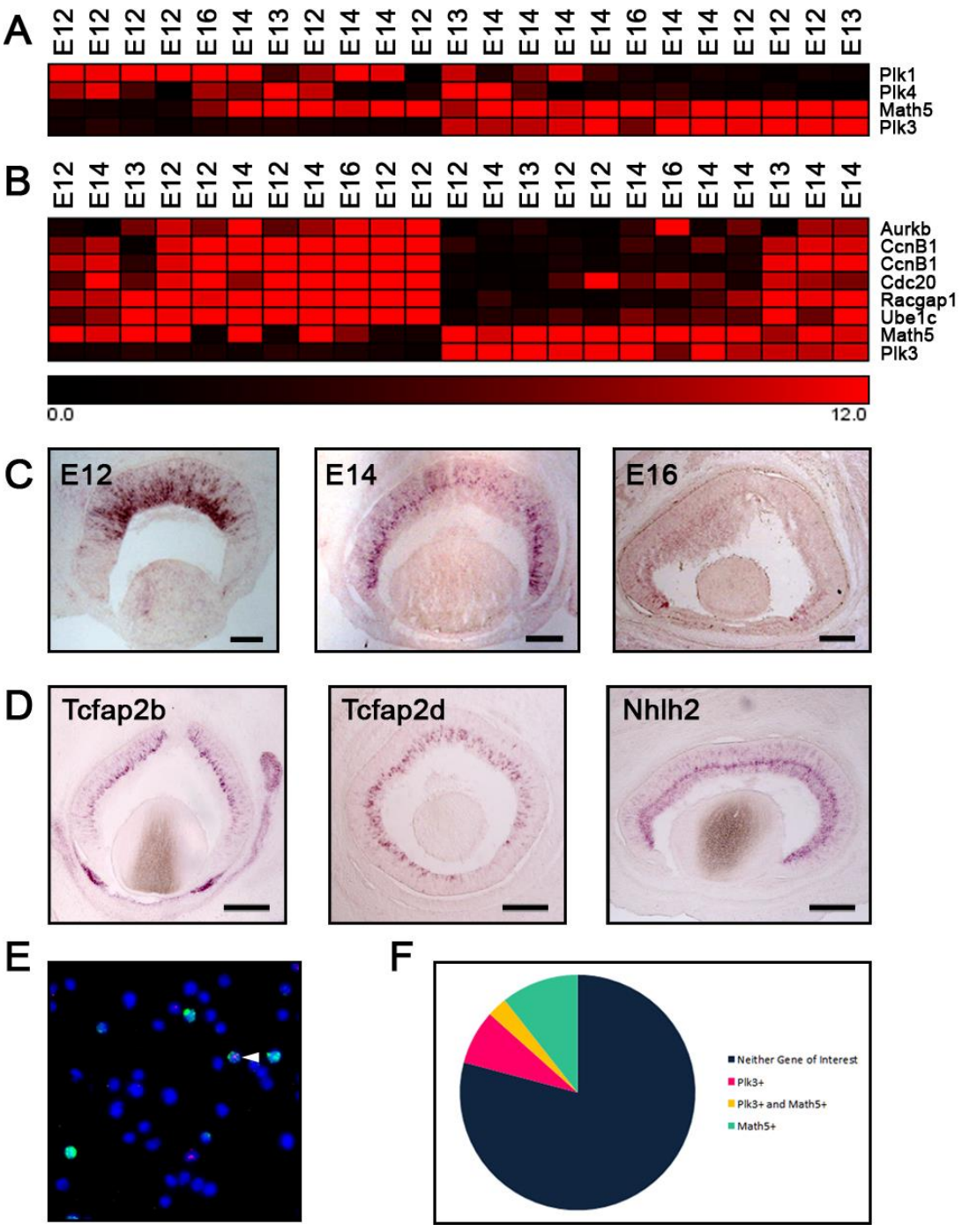

Figure 1. Survey of Plk3 expression in the retina

Single-cell transcriptomic data indicated that Plk3 was among the genes with the highest correlation with Math5, a marker of early-born retinal cells, during retinogenesis. (A) Comparisons of Plk3 to family members expressed in the retina, including Plk1 and Plk4, indicate that it does not segregate with its family members.

(B) Further comparisons of cells expressing Math5 and Plk3 with other embryonic cells determined that Plk3, which has known roles as a cell cycle regulator, also does not correlate with markers of cycling cells, suggesting another role for this kinase in retinal development. (C) Confirmation of Plk3's expression in the developing retina indicates strong expression in the neuroblast layer (NBL) at E12, which follows the wave of proliferation of retinal progenitors at E14 and subsides to undetectable levels throughout the retina by E16. (D) The expression patterns of Plk3 are reminiscent of markers of subsets of amacrine interneurons, including Tcfap2b, Tcfap2d, and Nhlh2, at E14. (E) Double fluorescent *in situ* hybridization was employed to understand the overlap in expression between Math5 and Plk3 at E14, which was quantified in (F). All scalebars represent 100 μm.

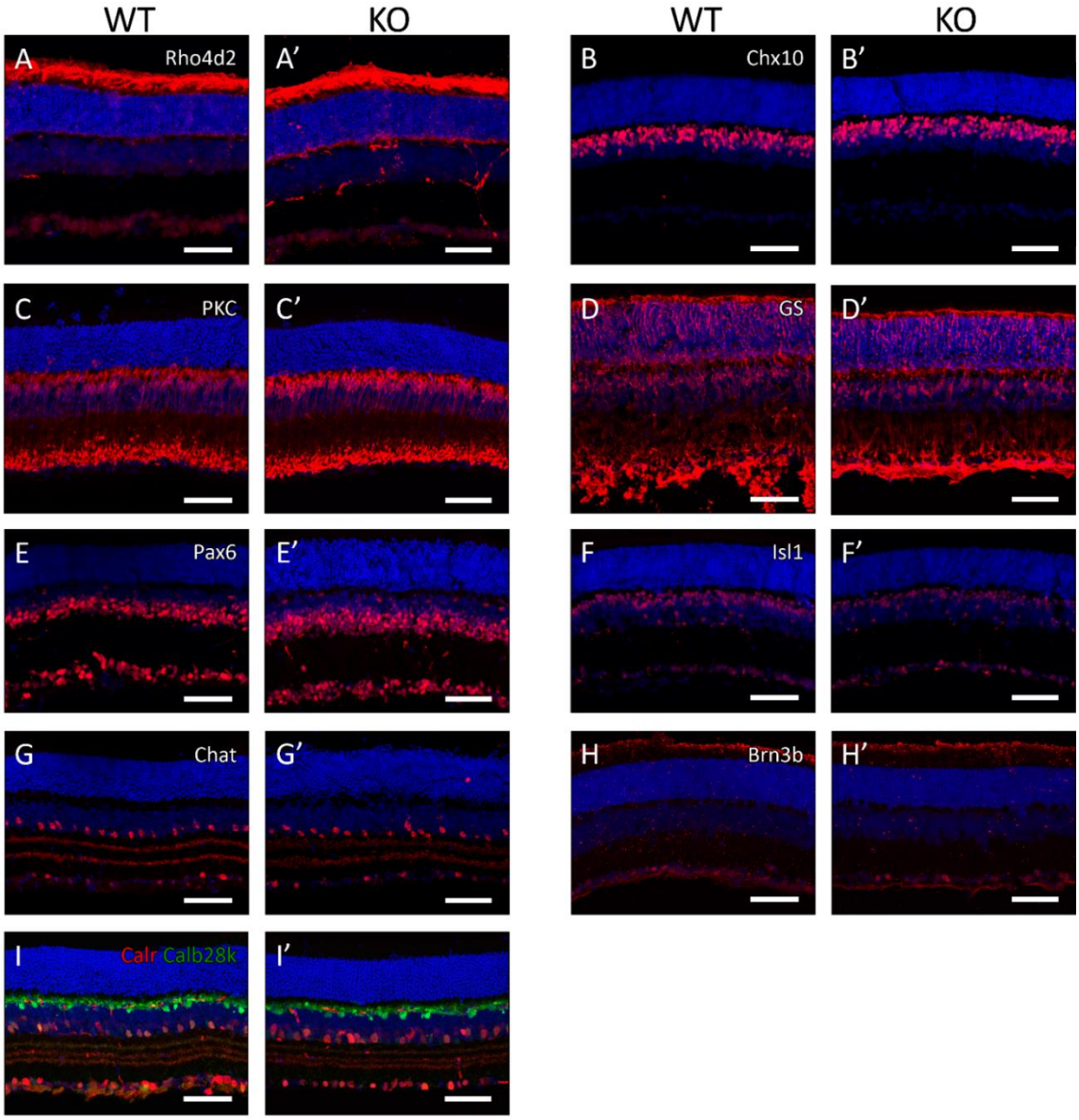

Figure 2. Morphological characterization of adult Plk3-KO retinas

Populations of adult retinal cells were identified using antibodies to neuron-specific markers. DAPI, in blue, marks nuclei. Populations of photoreceptors (Rho4d2, A,A'), bipolar interneurons (Chx10, B,B'; PKC, C,C'), Muller glia (GS, D,D'), amacrine interneurons (Pax6, E,E'; Isl1,F,F'; Chat, G,G'; Calretinin,Calb28k, F,F'), retinal ganglion cells (Brn3b, H,H'), and horizontal interneurons (Calr,Calb28k, I,I') were not reproducibly affected by loss of Plk3 during development. Scalebars represent 100 μm.

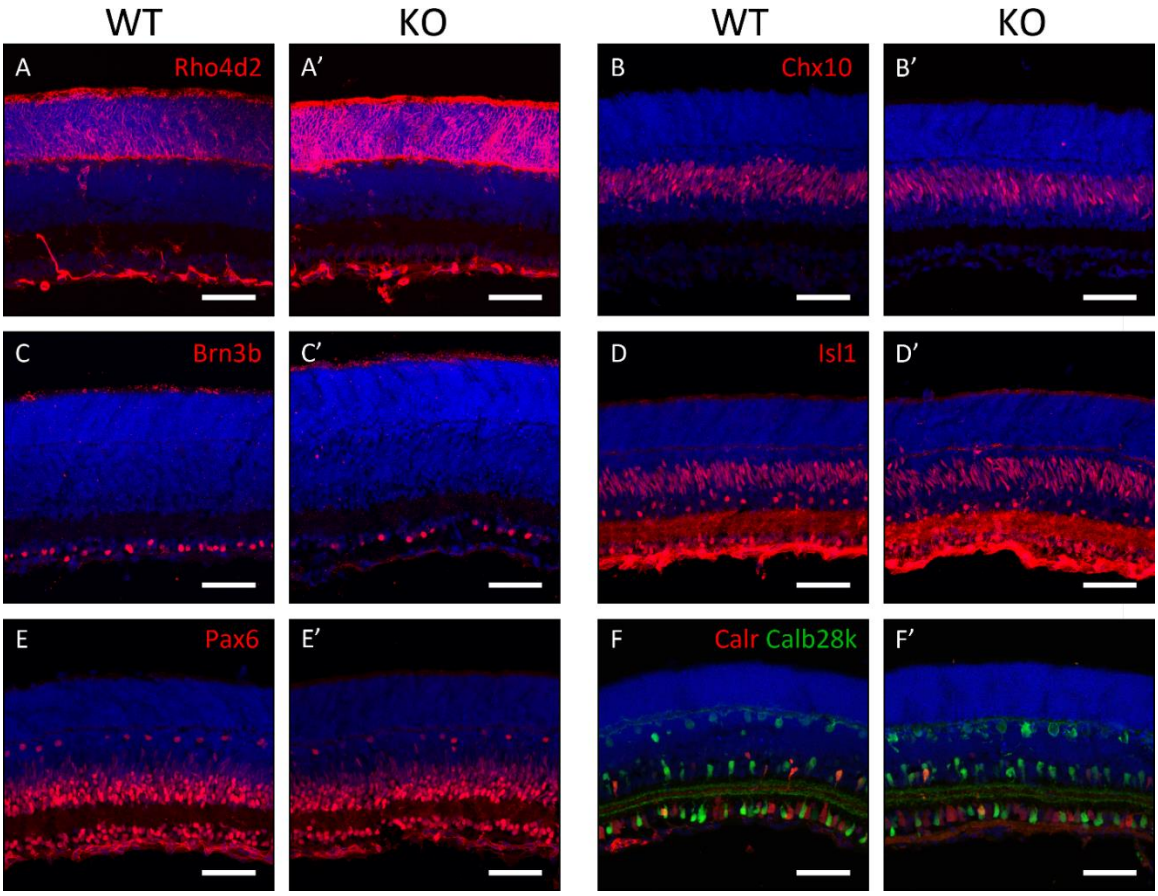

Figure 3. Morphological characterization of developing Plk3-KO retinas

Populations of retinal cells in postnatal day (P)7 Plk3-KO retinas were compared to WT littermates using cell type-specific markers, indicating few changes upon loss of Plk3 during development in populations of photoreceptors (Rho4d2, A,A'), bipolar cells (Chx10, B,B'), retinal ganglion cells (Brn3b, C,C'), amacrine interneurons (Isl1, D,D'; Pax6, E,E'; Calr, Calb28k, F,F'), or horizontal cells (Calr,Calb28k, F,F'). Scalebars indicate 100 µm.

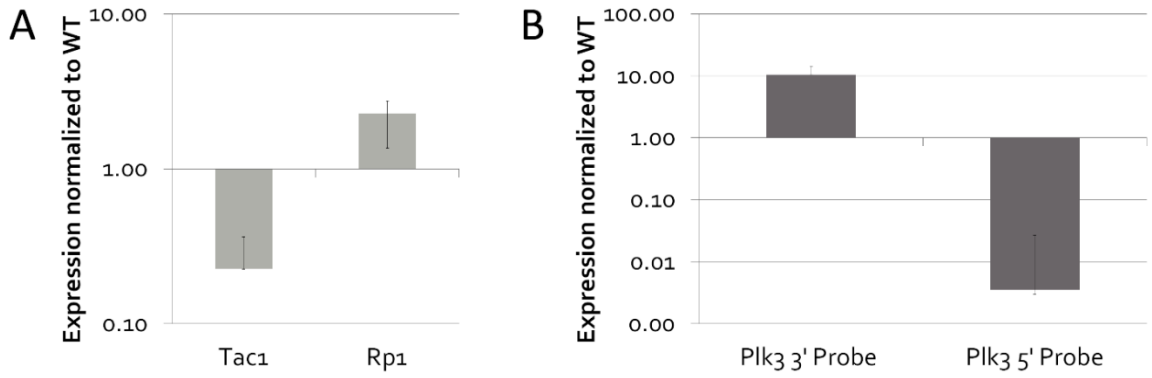

Figure 4. Confirmation of unbiased screens for transcriptomic changes in retinal populations

Retinas of Plk3-KO and WT littermates were isolated and hybridizaed to Affymetrix microarrays at various timepoints (n=3 for each of four timepoints). Genes with significant differential expression at multiple timepoints were confirmed using qPCR at adult timepoints. (A) Tac1, a marker of a subtype of amacrine cells, was confirmed to have significantly decreased expression ($p<0.05$), whereas retinitis pigmentosa 1 (Rp1), a gene found in photoreceptors, was significantly increased ($p>0.05$). (B) Interesting, one of the significantly overexpressed genes on microarrays in Plk3-KO mice at all ages was Plk3 itself. This was due to upregulation of the 3' end of the gene, to which the microarray probe is designed, as confirmed by qPCR ($p<0.001$). However, the 5' end of Plk3 that was excised in the production of the Plk3-KO mouse was significantly downregulated ($p<0.05$).

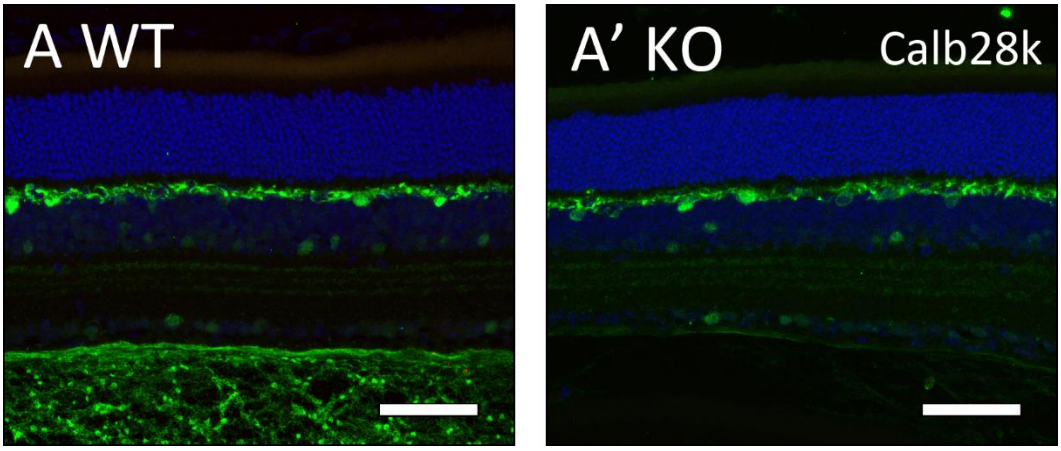

Figure S1. Examination of potential IPL phenotype using paraffin sectioning.

Initial surveys of retinal morphology at multiple timepoints suggested a possible phenotype in the organization of the IPL, especially as indicated through Calb28k staining. While present in multiple retinas, the disorganization of the IPL was not readily reproducible. Paraffin sectioning confirmed a lack of phenotype between WT (A) and adult Plk3-KO retinas (A'). Scalebars indicate 100 μm.

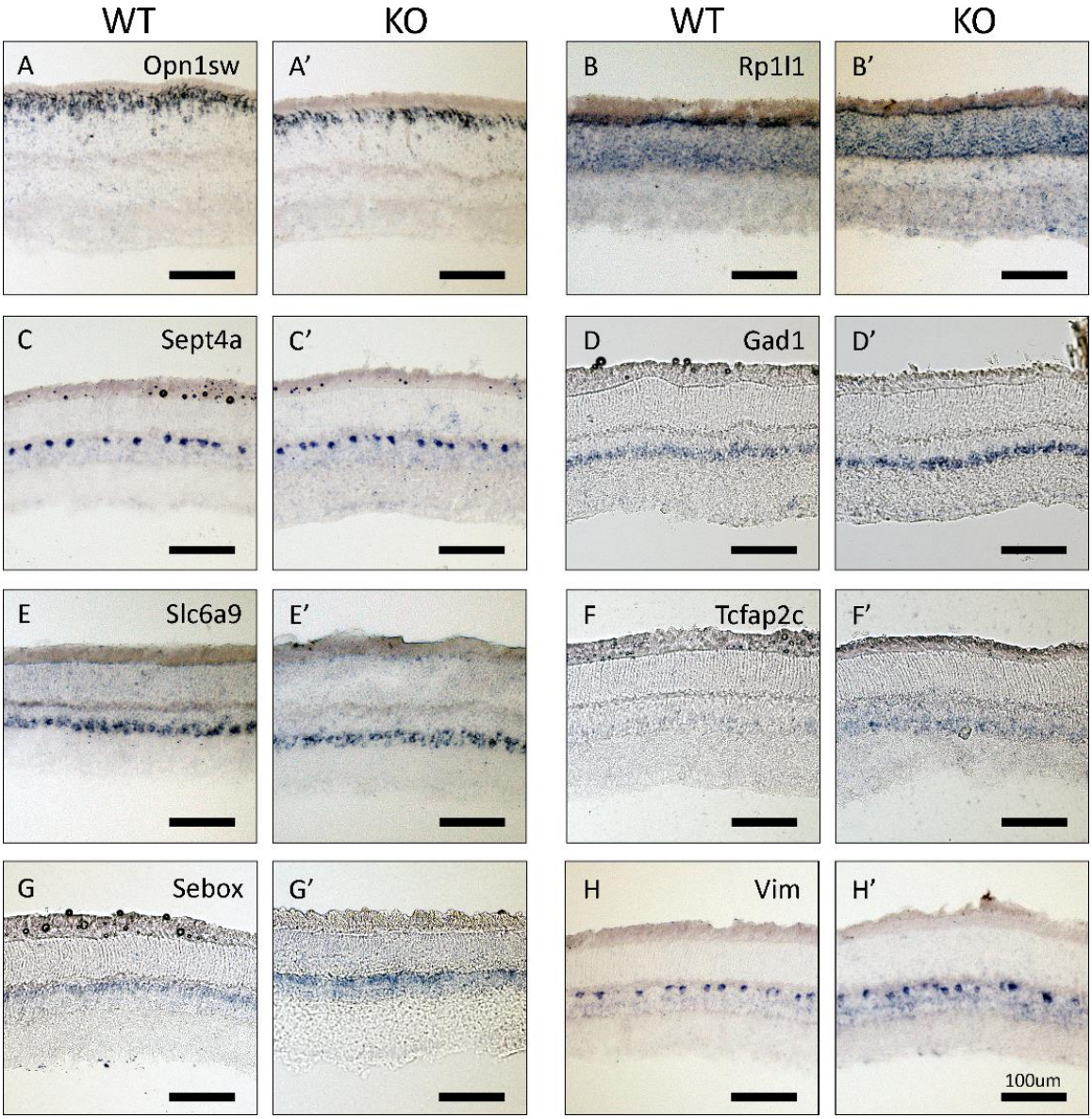

Figure S2. In situ hybridization of subsets of retinal cells in the adult Plk3-KO retina.

Differences in subsets of neuronal populations between WT and Plk3-KO retinas were visualized using *in situ* hybridization. Significant differences were not observed in populations of short-wave cones (Opn1sw, A, A'), photoreceptors (Rp1l1, B,B'), or subsets of amacrine cells (Sept4, C,C'; Gad1, D,D'; Slc6a9, E,E'; Tcfap2c, F,F'; Sebox, G,G'; Vimentin, H,H'). Scalebars represent 100 μm.

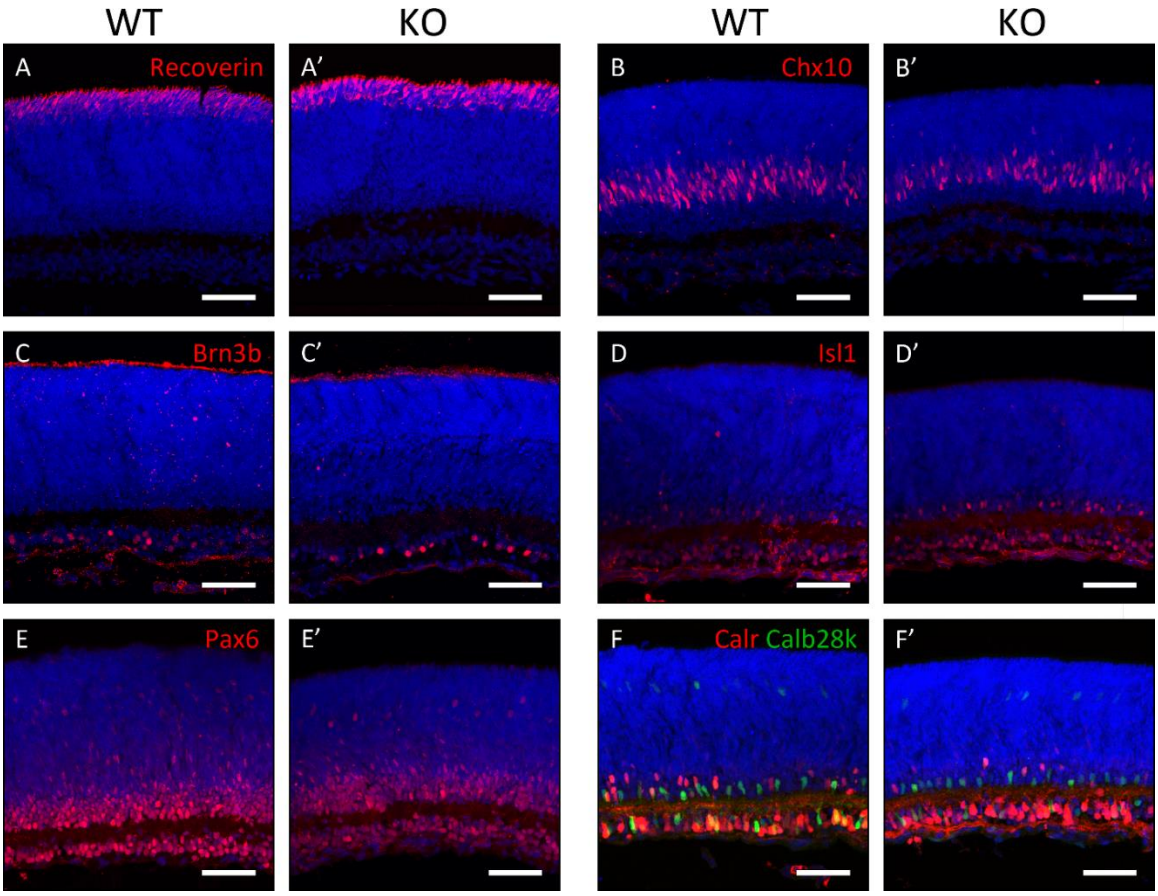

Figure S3. Survey of retinal morphology in the newly postnatal (P4) Plk3-KO retina.

Populations of retinal cells in P4 Plk3-KO retinas were compared to WT littermates using celltype-specific markers, indicating few changes upon loss of Plk3 during development in populations of photoreceptors (Recoverin, A,A'), bipolar cells (Chx10, B,B'), retinal ganglion cells (Brn3b, C,C'), amacrine interneurons (Isl1, D,D'; Pax6, E,E'; Calr, Calb28k, F,F'), or horizontal cells (Calr,Calb28k, F,F'). Scalebars indicate 100 μm.

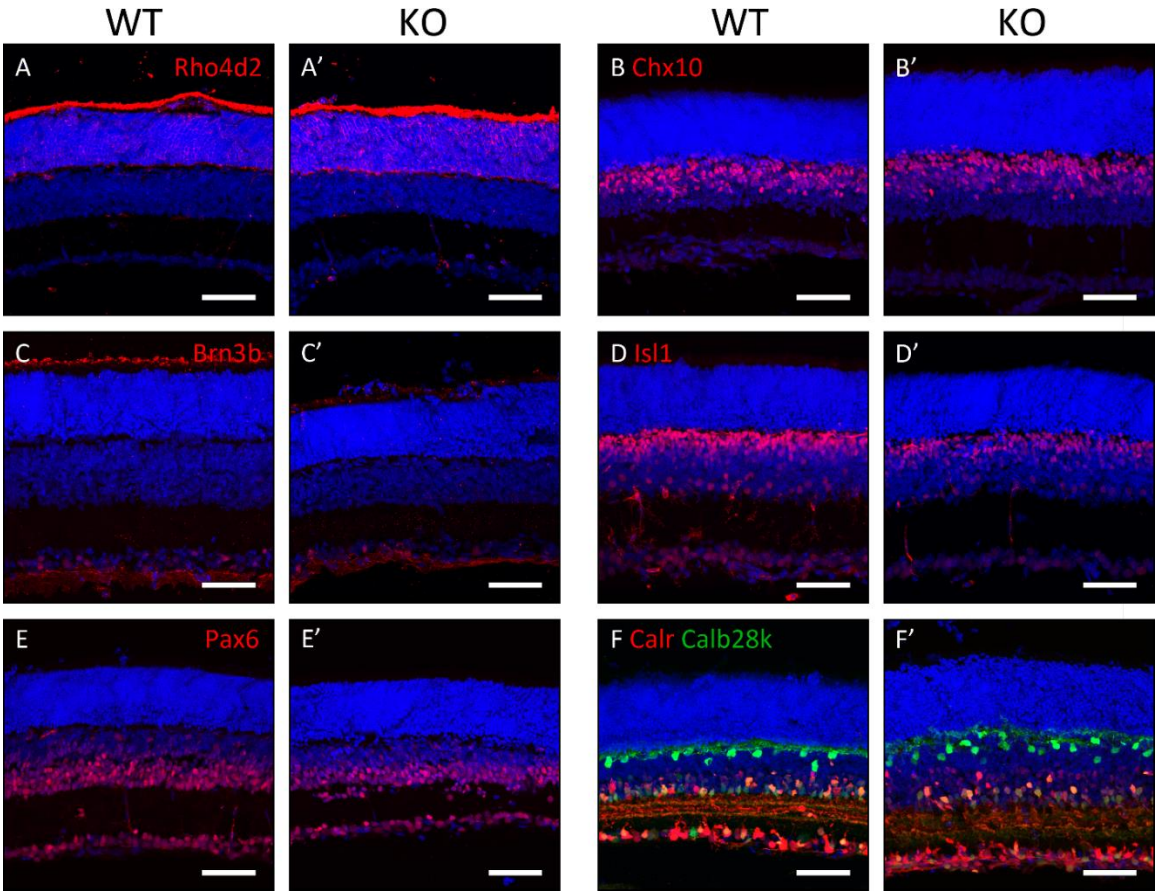

Figure S4. Survey of retinal morphology in nearly mature (P14) Plk3-KO retina.

Populations of retinal cells in P14 Plk3-KO retinas were compared to WT littermates using celltype-specific markers, indicating few changes upon loss of Plk3 during development in populations of photoreceptors (Rho4d2, A,A'), bipolar cells (Chx10, B,B'), retinal ganglion cells (Brn3b, C,C'), amacrine interneurons (Isl1, D,D'; Pax6, E,E'; Calr, Calb28k, F,F'), or horizontal cells (Calr,Calb28k, F,F'). Scalebars indicate 100 μm.

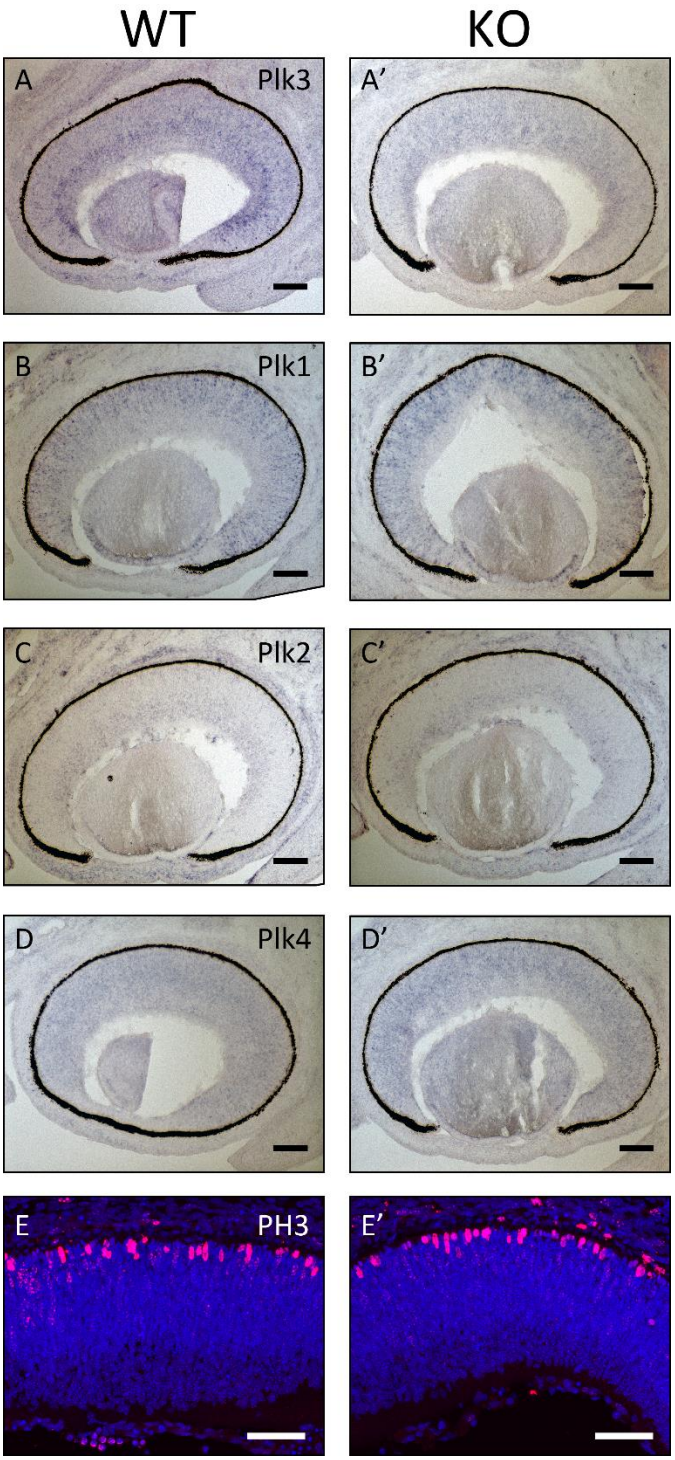

Figure S5. Survey of Plk family member expression patterns and cell cycle between WT and Plk3-KO retinas in mid retinogenesis

To determine whether a lack of phenotype resulting from loss of Plk3 mRNA (observable in A, A') was due to recovery from Plk family members, the expression patterns of Plk1 (B,B'), Plk2 (C,C'), and Plk4 (D,D') were determined using ISH at

E14. Although Plk3 expression levels are down as expected, other Plk family members do not appear changed between WT and Plk3-KO littermates. Further, to determine whether a phenotype existed in cell cycle maintenance, developing retinas were stained using αPH3 to mark mitosis (E,E'), but again no defects in could be identified resulting from loss of Plk3.

CHAPTER 4. SINGLE CELL TRANSCRIPTOME PROFILING OF DEVELOPING CHICK RETINAL NEURONS

1. Abstract

The vertebrate retina is a complexly-functioning photosensitive tissue comprised of six main neuronal and one glial cell populations, each of which develops in prescribed proportions at overlapping timepoints from a common progenitor pool. While each of these cells has a specific function contributing to proper vision in the mature animal, their differential representation in the retina as well as the presence of distinctive cellular subtypes makes identifying the transcriptomic signatures that lead to each retinal cell's fate determination and development difficult. We have analyzed transcriptomes from individual retinal cells isolated from the chicken throughout retinogenesis. These developing chicken cells are representative of multiple retinal cell types, including photoreceptors, interneurons, and retinal ganglion cells at various stages of development. A comparative analysis of the gene expression patterns found in these chicken retinal cells with previous mouse retinal cells reveals commonalities in vertebrate retinogenesis throughout evolutionary time. Taken together, these datasets will enable us to uncover the most critical genes acting in the steps of cell fate determination and early differentiation of various retinal cell types.

2. Introduction

Although interest in vertebrate neurogenesis is inevitably tied to the diversity of mature neurons, their connections, and specialized functions, that same complexity makes the system extremely difficult to study and understand. Through combinations of intrinsic and extrinsic signaling within neural progenitor populations, specific neuronal types with distinct functionalities must be generated in the proper numbers at the correct timepoints. Parsing through the transcriptomic and intracellular signals that drive the formation of a fully operational central nervous system (CNS) is a daunting task. Fortunately, the intricacies of CNS development can be modeled through the genesis of the relatively simple and easily-isolated neural retina. Vertebrate retinas are well-conserved and consist of organized layers that include six neural cell types and one type of glia. Among these neurons are cone and rod photoreceptors, arrayed in the apical outer nuclear layer; the interneurons of the inner nuclear layer, including amacrine, horizontal, and bipolar cells, as well as Muller glia; and the retinal ganglion cells (RGCs), comprising the retina's sole output to the brain, with nuclei located in the basal ganglion cell layer (Masland, 2001).

However, even a relatively simple model of neurogenesis has its complications. Each retinal cell is generated from a common population of retinal progenitor cells (RPCs) in distinct yet overlapping periods during retinogenesis (Young, 1985). In particular, the timespans during which chicken RPCs are competent to generate specific fates could be anywhere from a few days to over a week (Calaza Kda and Gardino, 2010). To add to this complexity, RPCs have been

shown through lineage analyses to be multipotent, capable of generating various combinations of retinal cell types (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). A last consideration when analyzing the ways that RPCs differentiate within the developing retina is the relative abundance of critical cell types that those RPCs generate. For instance, although RGCs are the only retinal cells that communicate visual signals to the brain, and can be further divided into upwards of 20 functionally distinct subtypes (Rockhill et al., 2002), they tend to make up a relatively small fraction of the total amount of retinal cells (Dräger and Olsen, 1981; Young, 1985). With all these caveats in mind, it is easy to imagine the shortcomings of using whole- or partial-retina studies to better understand the cell-intrinsic signals that drive each individual RPC towards its specific fate on its own timeline. For these reasons, we have taken a single-cell approach to differentiate between progenitors throughout retinogenesis.

Single-cell transcriptomic approaches have determined that individual mouse RPCs and the newly-born neurons they generate display extensive heterogeneity even at the same timepoints and as they generate similar types of cells (Cherry et al., 2009; Trimarchi et al., 2008). Although studies in the mouse have revealed much that can be applied to human development, the mouse does have its limitations. Therefore, the current study focuses on exploring retinogenesis at single cell resolution in the chicken for a variety of reasons. The retina's functionality as the origin of visual signals means that the selection of a model organism has unique considerations. The diurnal chicken relies more on its vision than the nocturnal mouse, and its retina shows many similarities to humans as a result. For instance,

the chicken's cone opsins have spectra with greatest sensitivity within the human visual spectrum (Mey and Thanos, 2000), as opposed to the mouse, whose two cones are most sensitive at 360 (S-opsin) and 580 nm (M-opsin) (Fu and Yau, 2007). The chicken also develops rapidly, with its large optic cups noticeable and accessible by embryonic day (E)3, and hatching by E21. The ease of manipulation of chick embryos *in ovo* is also a major advantage over mammalian model systems, such that multiple techniques for gene overexpression and knockdown analysis are highly refined (Vergara and Canto-Soler, 2012). Although the genome is fully sequenced and our understanding of the mature chicken retina continues to expand (Naito and Chen, 2004; Vergara and Canto-Soler, 2012), comparatively little is known about the development of the chick retina, especially at the level of individual progenitors.

Here we describe the transcriptomes of individual developing chicken retinal cells at multiple developmental timepoints. We have determined many clusters of coexpressed genes that define different states of RPC development. Overall, these clusters compare favorably to our previously generated transcriptomes of developing mouse cells. Although our initial focus was to determine the genes that drive the development of RGC, the critical output neurons of the retina, we also identified individual cells at various stages of maturation into amacrine and horizontal interneurons, as well as cones. From these transcriptomes, we have learned much about the gene expression of developing chick retinal cells, which can be compared with our mouse data to define the most highly conserved gene expression modules.

3. Materials and methods

Single cell microarrays

Tissue dissociation. For any steps in the single cell protocol requiring trituration or addition of reagents, filter tips were employed to avoid potential contamination. Briefly, retinas were isolated and placed in a 1.5 ml microcentrifuge tube containing dissociation solution (380 µl Hank's Balanced Salt Solution [HBSS] supplemented with 10 mM HEPES, 10 µl 25 mM Cysteine in 5 mM EDTA pH 8.0, and 10 µl papain). The mixture was incubated for 5 min at 37°C, after which, any settled tissue was dislodged by firm tapping. To insure complete dissociation, if clumps remain the tissue can be further agitated by gentle trituration 10-15 times using a p1000 pipettor. 5 µl of DNase I was added to remove the genomic DNA, and the samples were again incubated for 5 min at 37°C. After further trituration, the samples were centrifuged for 3 min at 3000 rpm. The supernatant was carefully removed to a final volume of between 100-200 µl, and the pellet of dissociated cells dislodged by firm tapping. 1 ml of HBSS was used to further resuspend the dislodged pellet. Samples were again centrifuged for 3 min at 3000 rpm before as much supernatant as possible was removed in order to minimize contamination from lysed cells and overdissociation from trace amounts of papain. Samples were then resuspended in 1 ml of 1X PBS supplemented with 0.1% BSA.

Single cell isolation. Single-cell isolation was performed as described (Goetz and Trimarchi, 2012). A portion of the 1 ml of resuspended cells was plated onto a 6 cm dish containing 5 ml of PBS/ 0.1% BSA and allowed to settle (about 5 min). Due

to the large size of chicken retinas, the exact portion of the final volume of the dissociated sample to be plated was empirically determined for each developmental timepoint. Cells were isolated on an Olympus IMT-2 inverted microscope, using 38.1 cm long aspirators (Sigma) tipped with pulled-glass micropipettes (inner diameter 0.5 mm, outer diameter 1.04 mm). While cells readily entered the micropipette through capillary action, they were gently expelled using pressure on the aspirator tube. After initial cell selection on the first plate, isolation of an individual cell and contamination minimization was ensured by use of a second "wash" 6 cm dish filled with 5 ml 1X PBS/ 0.1% BSA. A fresh micropipette was always employed to move an individual cell from the wash plate to a 0.2 ml PCR tube containing 4.5 µl of cell lysis buffer. In an attempt to maximize the number of developing RGCs isolated, cells were selected based upon their size (i.e. the largest cells were targeted for isolation).

cDNA preparation. After isolation, samples were briefly spun in a table-top microcentrifuge to ensure submersion of cells into the lysis buffer. Lysis was promoted by incubating the samples in a thermocycler at 70°C for 90 sec before immediate replacement on ice. Reverse transcription mixture (0.33 µl SuperScript III [200 U/µl], 0.05 µl RNase Inhibitor [40 U/µl], and 0.07 µl T4 gene 32 protein) was added, and the mixture incubated at 50°C for 50 minutes and inactivated at 70°C for 15 minutes before replacement on ice. Free primer removal was accomplished by adding 0.1 µl of Exonuclease I, 0.1 µl of 10X Exonuclease I buffer, and 0.8 µl of molecular biology grade water to the samples before incubating at 37°C for 30 min and inactivating at 80°C for 25 min. Tailing reaction mixture (0.6 µl 10X PCR Buffer,

0.18 µl 100 mM dATP, 0.3 µl TdT, 0.3 µl RNase H, and 4.62 µl molecular biology grade water) was added to the samples, which were incubated at 37°C for 20 min and inactivated at 70°C for 10 minutes. Finally, single-cell PCR mixture (10 µl 10X ExTaq Buffer, 10 µl 2.5 mM dNTPs, 0.2 µl 100 µm OligoDT, 1 µl TaKaRa ExTaq, and 65.7 µl molecular biology grade water) was added to samples. The PCR reaction used to amplify single-cell cDNA is as follows: 95°C for 2 min; 37°C for 5 min; 72°C for 16 min; then 34 repetitions of 93°C for 40 sec, 67°C for 1 min, and 72°C for 6 min plus 6 sec more per cycle; finally, after incubating at 72°C for 10 min, the samples may be held overnight at 4°C.

Testing cDNA quality. 10 µl of cDNA smears were run on a 1% agarose gel along with a DNA size ladder to determine cDNA library quality. Ideal libraries exhibited a robust smear most brightly between 500-2000 bp. These were compared to blank media samples that were run through the entire washing and amplification process to determine whether any contamination exists throughout the process. Further quality tests to pre-screen samples before microarray hybridization included PCR screens for specific marker genes.

cDNA fragmentation and microarray hybridization. 15 µg of (1 µg/µl) amplified cDNA samples was fragmented in a solution containing 8 µl 1X One-Phor-All Buffer, 1 µl of DNaseI (diluted 1:10 in 1X One-Phor-All Buffer). Samples were incubated at 37°C for 13 min and inactivated at 99°C for 15 min. Labelling was accomplished by adding 20 µl 5X TdT buffer, 2.5 µl Biotin N6-ddATP (Enzo Biosciences), and 1 µl TdT (diluted 1:8 in TdT buffer), then incubating at 37°C for 90

min and inactivated at 65°C for 5 minutes, before storage at -20°C or immediate hybridization to Affymetrix microarrays according to standard Affymetrix protocols.

Clustering/heatmaps

Clusters of co-expressed genes were determined using hierarchical clustering through the Gene Cluster Software package (Eisen et al 1998). The data were filtered by signal such that any gene not achieving a signal of 1000 in at least one single cell was removed. The remaining data was log-transformed and normalized using the standard methods included with the software (Eisen et al., 1998). Heatmaps were generated using Genesis (Thallinger Lab, Graz University of Technology, Austria).

***In situ* hybridizations**

Riboprobe synthesis. Sequences for probe templates were amplified from chicken cDNA and cloned into Promega pGEM-T vectors before confirmation via sequencing. Depending on the direction of insertion, antisense riboprobes were then transcribed using T7 or Sp6 RNA polymerase in the presence of digoxigenin (DIG)-labeled nucleotides for 1 hour at 37°C. After a 15-minute DNase I treatment at 37°C, probes were precipitated overnight using LiCl and 100% Ethanol.

In situ *hybridization.* In situ hybridizations on tissue cryosections and dissociated cells were performed as previously described (Trimarchi et al., 2007).

3. Results and discussion

Our research has previously focused on elucidating the cell-intrinsic mechanisms driving neurogenesis in the developing murine retina using single cell transcriptomic profiling. Through our analysis of over 200 cells from various stages of development, we were able to identify novel markers of retinal progenitor cells (RPCs) and maturing retinal cells as well as differentiate between developing subsets of retinal cell types (Cherry et al., 2009; Trimarchi et al., 2007, 2008). Since our single-cell isolation techniques have proven to be a robust method for transcriptomic analysis in the mouse, we sought to expand our studies beyond that original model system for a variety of reasons. For instance, although the mouse is a well-studied and understood genetic model, it may not be ideal for retinal studies in particular. The nocturnal mouse does not rely primarily on its vision and thus has a less-developed visual system than diurnal animals, so that extrapolating the transcriptomic signatures of developing cell types to human studies may be more straightforward in other systems. Additionally, regardless of the mouse's suitability as a model system, the addition of an evolutionarily divergent model system into our database of developing cells can aid in differentiating between peculiarities of development in either model system and better focusing our future studies on those genetic markers that are conserved throughout evolution as an indicator of greater functional relevance, as previously described in studies of neural crest development (Barriga et al., 2015).

The developing chicken is an excellent model system for retinogenesis studies not only for its ease of care and large, accessible retinas, but also for the

diurnal chicken's greater reliance on cone-driven vision, which much more closely parallels the human retinal organization than the nocturnal mouse. For these reasons, we have isolated chicken retinal cells from various embryonic timepoints as previously described (Goetz & Trimarchi, 2012). Although our initial goal was to isolate larger cells from dissociated retinas to gain more insights into RGC development in particular, we were able to identify a sampling of multiple neuronal subtypes at various stages of development. We can extrapolate information from previous studies based on the chick visual system as well as our own database of individual cells to retrospectively identify cells isolated by visual identification.

Insights from single retinal cells

After isolation and cDNA amplification, each cell in this study underwent preliminary PCR screening for markers of specific retinal cells. These screens served the dual purpose of ensuring samples were pure representations of genes expressed in a single retinal cell, as well as allowing for an initial characterization of the exact type of cell isolated. Of the 14 cells isolated for this study, one in particular [Embryonic day (E)4 Cell #1] stood out as being Cath5-positive, indicating its identity as a progenitor around the time of its final division (Feng et al., 2010; Le et al., 2006). Upon further examination, we observed that this cell also expresses Fgf19, a marker of a subset of progenitors differentiating into horizontal cells (Figure 1A) (Okamoto et al., 2009), indicating that this cell is potentially a developing HC. To better understand Fgf19's role as a marker of horizontal cells, we designed an RNA probe to identify Fgf19's expression patterns using in situ

hybridization (ISH) at E4 (Figure 1B), and E5, E7, and E8 (Figure 1C). We observed that Fgf19 marks a subset of cells in the progenitor layer during early retinogenesis (E4-E5), consistent with previous studies that indicate horizontal cells are differentiating and migrating between the apical and basal poles of the retina between E4.5-E8 (Edqvist and Hallböök, 2004). To confirm that these cells were cycling progenitor cells, we introduced 3H-thymidine *in ovo* at E4 for 1 hour before isolating the retina. In situ hybridizations for Fgf19 performed in conjunction with 3H-thymidine detection demonstrated a 22% overlap at E4 after a 1 hour pulse. This shows that many Fgf19 expressing cells are cycling progenitor cells. By E7, Fgf19 expression is focused mainly at the basal portion of the retina, where a two-day arrest of horizontal-fated RPCs has been noted to occur before their final mitosis (Boije et al., 2013). By E8, Fgf19 expression is confined to the horizontal cell layer, indicated by a black arrowhead.

We looked further at the transcriptome of E4 Cell #1 and found additional HC marker genes (Onecut2 and Lhx1), but also genes that are expressed in developing cone photoreceptors (Otx2, NeuroD1 and Sall1). While initially surprising, this interesting finding could be tied back to our studies of the Onecut family of transcription factors. Initially discovered in subsets of developing murine single cells, studies of Onecut1- and Onecut2-deficient mice revealed that these genes are required for the generation of horizontal cells (Goetz et al., 2014). However, these genes are not purely limited to horizontal cell expression, as further investigations indicate that Onecut1 is a part of a regulatory network downstream of the thyroid hormone receptor beta (Thrb) gene, which biases retinal progenitors towards

assuming either a horizontal or cone photoreceptor fate (Emerson et al., 2013). Once Thrb is expressed in a retinal progenitor, greater combinatorial expression of the transcription factors Onecut1 or Otx2 weigh the progenitor towards either a horizontal or cone fate, respectively (Emerson et al., 2013). These intriguing findings corroborate the identity of E4 Cell #1 as a progenitor near the cusp of determining its fate as a horizontal cell or a cone photoreceptor, and underscore the importance of transcriptomic analysis at the single cell resolution. Furthermore, given where this cell stands in terms of cell fate determination, it is interesting to note the abundance of transcription factors (Nr6a1, Pbx4, Olig3, Stat3, Beta3 and Zic1) present in this single cell. Determining how all these transcription factors work together to produce a specific progeny cell will be a challenge for future studies.

Analysis of isolated retinal ganglion cells

Among the other early-developing retinal cells are retinal ganglion cells (RGCs), the sole output neuron connecting the retina to the rest of the visual system. As our initial goal was to isolate these critical neurons, we initially sought to isolate cells with above-average sized somas from dissociated retinas. To determine which of the isolated cells might be developing RGCs, we used unsupervised hierarchical clustering to cluster the genes and the cells. We then searched for established markers of RGCs, such as Uchl1, NF-M, and NF-L (Figure 2A) (Trimarchi et al., 2007), to pinpoint which cells in our dataset were developing RGCs. We sought to better understand the expression patterns of these genes that were highly correlated with

known RGC markers in early retinogenesis in the chicken retina. ISH probes were designed to genes highly expressed in the putative RGCs, including Uchl1 (Figure 2B), Ppp3ca (Figure 2C), and Tagln3 (Figure 2D). Composites of the full chicken retina at E4 show that the genes from our possible RGCs are indeed present in the developing retina in a pattern suggestive of being exclusive to developing RGCs (Figure 2B-D, see arrowheads indicating expression in the developing retinal ganglion cell layer).

With confirmation that the single cells identified in Figure 2A were bona fide developing RGCs, we sought to further elucidate the expression patterns of genes expressed exclusively in those cells throughout retinal development. Hierarchical clustering was performed to generate an unbiased cluster of the genes most closely associated with known RGC markers. After clustering, the expression pattern of genes highly correlated with known RGC-related genes Uchl1 (Figure 3A-A'''), NF-L (Figure 3B-B'''), and NF-M (Figure 3C-C''') were determined through ISH at various timepoints between early retinogenesis and later stages of development as labeled. It is important to note that while fully sequenced, the chicken genome is not completely annotated. Some genes that clustered closely with RGC-specific genes therefore had generic names, such as 382L11 (3D-D'''). When ISH of genes such as this one indicated high specificity for RGCs, we further investigated the sequence of the unknown gene to determine that it was highly homologous to a known RGC marker from other species, Brn3a. While other genes with high correlation to these markers of RGCs were themselves previously associated with RGC development, such as Ebf3 (Figure 3E-E'''), others were not previously explored in conjunction

with RGC development or maintenance in particular (Tagln3, Figure 3F-F'''; Robo2, Figure 3G-G'''; 742d11, Figure H-H'''). Many of the genes seen in early-developing RGCs were confined to the inner neuroblastic layer (INBL) at early timepoints (Figure 3A, C, E, F, H), while others exhibit more generalized expression in early retinogenesis (Figure 3B). While many genes eventually resolved to various retinal populations, the RGC layer was generally among the highest areas of late embryonic expression for each gene. Uchl1, Nf-m, Ebf3, Tagln3, Robo2, and 742d11 (Figure 3A''', C''', E''', F''', G''', H''') mark both subsets of cells in the RGC layer and varying numbers of cells in the INL, which could be either interneurons or possibly displaced RGCs (Naito & Chen, 2004). Other genes, such as NF-L and 382L11 (Figure 3B, D), appear to resolve with greater exclusivity to the RGC layer by late retinal development (Figure 3B''', D'''). This cluster of genes that is strongly correlated with the neurofilaments also contains many uncharacterized ESTs (data not shown). It will be of great interest in the future to identify the precise nature of these ESTs and determine whether they have counterparts that are also expressed during mouse RGC development.

Since part of our interest in expanding our studies of single-cell transcriptomics to a model system beyond the mouse was to compare and contrast the differences in transcriptomes throughout retinogenesis among vertebrates, we turned back to previous studies (Trimarchi et al., 2007) to see the variation that existed between genes present in the developing chicken and mouse retina. We were interested in further exploring the expression of transcription factors that had been heterogeneously expressed in developing retinal cells in the mouse (Figure

4A). Somewhat surprisingly, transcription factors were under-represented in the cluster of genes that was strongly correlated with Nf-L and NF-M (data not shown). Although transcription factors in general were not particularly well-correlated in this dataset with known RGC markers including NF-L and NF-M, they were present in subsets of chicken retinal cells identified as RGCs (Figure 4A). We sought to further characterize these genes using *in situ* hybridization to identify the expression of these genes throughout retinogenesis (Figure 4B-E'''). While genes such as Scng and Isl1 are confined to the INBL during early stages of retinal development (Figure 4B, C), two family members of the Irx transcription factor family, Irx1 and Irx2, show expression in a subset of cells in the developing ganglion cell layer (Figure 4D, E). Most of these genes resolve their expression exclusively to the GCL by late retinogenesis (Figure 4B''', D''', E''') with the exception of Isl1 (Figure 4C''') which mirrors expression patterns in the mouse as a marker of not only cells in the GCL but also interneurons present in the INL. Therefore, it is possible that most transcription factors are only expressed initially in subsets of RGCs. This prediction will need to be tested by examining the different RGC subtypes in the chick in more detail.

Insights from isolated amacrine interneurons

Amacrine cells are the most diverse cells in the retina with various patterns of connectivity and neurotransmitters resulting in at least twenty subtypes being described in a multitude of different organisms (MacNeil and Masland, 1998). Given the variety of amacrine cells present in the retina, it is therefore expected that

determining markers that are both specific and generalizable among these varied interneurons has been a difficult feat. Markers of amacrine cells include transcription factors such as Pax6 and Tcfap2b (Cherry et al., 2009). At early timepoints, Pax6 is also expressed by cycling retinal progenitors, so Tcfap2b was used to classify E9 Cell #1 as an amacrine cell (Figure 5A). We sought to further characterize genes expressed in this cell by ISH to identify more amacrine cell markers, as well as to determine the subtype of amacrine cell E9 Cell #1 may be differentiating into, as well as those expressed in the cluster of E5 and E6 cells that also express known amacrine marker Isl1.

In situ hybridization of Tcfap2b in the chicken (Figure 5B-B’’’) indicated broader expression patterns that closely mirror those observed in mouse (Trimarchi et al., 2007). Although its expression is initially diffuse in the developing retina (Figure 5C), Slit2 was one of few genes with expression strictly limited to subsets of cells within the INL (Figure 5C-C’’’), leading us to believe that while it may be a specific marker of amacrines, it does not mark all amacrine populations. It is important to note upon observing the expression patterns of Tcfap2b, Tac1, and Clbn2 (Figure 5C-E, C’’’-E’’’) that around 50% of the cell bodies present in the GCL are actually displaced amacrine cells in some vertebrates (Masland, 2001), as exemplified by the resolution of expression of GABAergic amacrine marker Tac1 (Cherry et al., 2009) (Figure 5D-D’’’). Additionally, while the presence of neurofilaments in some potential amacrines (including E9 Cell #1, Figure 4A) may initially cause some pause, the neurofilament family has been observed to be present in subsets of amacrine cells (Trimarchi et al., 2007; Vaney et al., 1988).

While it is possible that these cells exhibiting markers of both amacrines and ganglion cells are cycling or newly-born progenitors that have not settled on a specific fate, the late stage at which these cells were identified is outside of the window for the birth of ganglion cells, which ends before the first embryonic week (Spence and Robson, 1989). This information, combined with previous observations of single cells in the mouse (Cherry et al., 2009; Trimarchi et al., 2007), led us to believe that these cells are indeed a diverse set of maturing amacrine cells.

Genes specific to cone photoreceptors identified in single cells

A mature cone cell was identified in the dataset (E15 Cell #1, Figure 6A) due to high expression of previously-determined photoreceptor-specific markers, such Otx2. We examined the transcriptional profile further and found other genes present in this cone cell that included Gnat2 (Figure 6B-B''), Rxrg (Figure 6C-C''), and Purpurin (Figure 6D-D''), the expression for each of which resolves to strong and exclusive expression in the ONL as expected for markers of cone cell. To determine the photoreceptive capabilities of E15 Cell #1, we studied the expression of the various opsins seen in chick cones and determined that this cell possesses low but significant expression of both violet opsin and the UV opsin Opn5 (Yamashita et al., 2014). These findings indicate that the isolated cell may be a specialized avian double-cone cell.

Previous studies of the individual transcriptomic differences that exist between cones in the chick have revealed some expected findings, such as the fact

that among the most highly differentially expressed genes among cone populations are their distinctive opsins (Enright et al., 2015). As confirmed by our previous single-cell studies (Trimarchi et al., 2008), transcription factors such as Sall1, Sall3 are also differentially expressed in cone populations along different timelines of expression (Enright et al., 2015). Receptors such as Rxrg and Thrb appear to be enriched in specific cone subpopulations (Enright et al., 2015). As all these genes are expressed in our own transcriptomic dataset, it will be interesting to compare and contrast our findings with their results so we can better aid in predicting the subset of cone that our progenitors will become. However, few conclusions can be made without the addition of more developing cones to our dataset. We are also interested in furthering our understanding of genes preferentially seen in newly developing progenitors as they decide upon a cone fate, such as E4 Cell #1(Figure 6A), a likely as-yet-uncommitted retinal progenitor, which was also mentioned as a possible horizontal cell.

4. Conclusions

The developing vertebrate retina requires an astonishing amount of coordination between progenitor cells to perform its highly complex and necessary functional role as a sensory tissue. While the retina functions properly only as the total sum of its parts, the development of each critical component – photoreceptor, ganglion cell, and interneuron alike – is just as critical as any other component, and must be observed at the resolution that decisions about cellular identity are made. Of course, the huge amount of transcripts present in any given progenitor, let alone group of progenitors on different steps towards differentiation to the same or different fates, is daunting to parse. For these reasons, we have expanded our studies from the transcriptomes of developing mouse retinal progenitors to another vertebrate model system, the chicken. In comparing the genes present in subsets of retinal cells throughout development, we are able to understand the factors in common between these two model systems that have maintained similar influences across evolutionary history. In this study we have determined many similarities in the transcriptomes between developing chicken and mouse retinal cells. As we expand our datasets to include more retinal cells, we hope to be able to eliminate the noise introduced by individual model systems to better understand the most important factors that lead to the specification of retinal cells among vertebrates.

5. References

Barriga, E.H., Trainor, P.A., Bronner, M., and Mayor, R. (2015). Animal models for studying neural crest development: is the mouse different? Development *142*, 1555–1560.

Boije, H., Shirazi Fard, S., Ring, H., and Hallbook, F. (2013). Forkheadbox N4 (FoxN4) triggers context-dependent differentiation in the developing chick retina and neural tube. Differentiation *85*, 11–19.

Calaza Kda, C., and Gardino, P.F. (2010). Neurochemical phenotype and birthdating of specific cell populations in the chick retina. Acad Bras Cienc *82*, 595–608.

Cherry, T.J., Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2009). Development and diversification of retinal amacrine interneurons at single cell resolution. Proc. Natl. Acad. Sci. *106*, 9495–9500.

Dräger, U.C., and Olsen, J.F. (1981). Ganglion cell distribution in the retina of the mouse. Invest. Ophthalmol. Vis. Sci. *20*, 285–293.

Edqvist, P.-H.D., and Hallböök, F. (2004). Newborn horizontal cells migrate bi-directionally across the neuroepithelium during retinal development. Dev. Camb. Engl. *131*, 1343–1351.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U A *95*, 14863–14868.

Emerson, M.M., Surzenko, N., Goetz, J.J., Trimarchi, J., and Cepko, C.L. (2013). Otx2 and Onecut1 Promote the Fates of Cone Photoreceptors and Horizontal Cells and Repress Rod Photoreceptors. Dev. Cell *26*, 59–72.

Enright, J.M., Lawrence, K.A., Hadzic, T., and Corbo, J.C. (2015). Transcriptome profiling of developing photoreceptor subtypes reveals candidate genes involved in avian photoreceptor diversification: Transcriptome Profiling of Avian Photoreceptors. J. Comp. Neurol. *523*, 649–668.

Feng, L., Xie, Z., Ding, Q., Xie, X., Libby, R.T., and Gan, L. (2010). MATH5 controls the acquisition of multiple retinal cell fates. Mol. Brain *3*, 36.

Fu, Y., and Yau, K.-W. (2007). Phototransduction in mouse rods and cones. Pflüg. Arch. - Eur. J. Physiol. *454*, 805–819.

Goetz, J.J., and Trimarchi, J.M. (2012). Single-cell profiling of developing and mature retinal neurons. J Vis Exp.

Goetz, J.J., Martin, G.M., Chowdhury, R., and Trimarchi, J.M. (2014). Onecut1 and Onecut2 Play Critical Roles in the Development of the Mouse Retina. PLoS ONE *9*, e110194.

Holt, C.E., Bertsch, T.W., Ellis, H.M., and Harris, W.A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron *1*, 15–26.

Le, T.T., Wroblewski, E., Patel, S., Riesenberg, A.N., and Brown, N.L. (2006). Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev Biol *295*, 764–778.

MacNeil, M.A., and Masland, R.H. (1998). Extreme diversity among amacrine cells: implications for function. Neuron *20*, 971–982.

Masland, R.H. (2001). The fundamental plan of the retina. Nat. Neurosci. *4*, 877–886.

Mey, J., and Thanos, S. (2000). Development of the visual system of the chick. Brain Res. Rev. *32*, 343–379.

Naito, J., and Chen, Y. (2004). Morphological features of chick retinal ganglion cells. Anat. Sci. Int. *79*, 213–225.

Okamoto, M., Bito, T., Noji, S., and Ohuchi, H. (2009). Subtype-specific expression of Fgf19 during horizontal cell development of the chicken retina. Gene Expr. Patterns *9*, 306–313.

Rockhill, R.L., Daly, F.J., MacNeil, M.A., Brown, S.P., and Masland, R.H. (2002). The diversity of ganglion cells in a mammalian retina. J. Neurosci. Off. J. Soc. Neurosci. *22*, 3831–3843.

Spence, S.G., and Robson, J.A. (1989). An autoradiographic analysis of neurogenesis in the chick retina in vitro and in vivo. Neuroscience *32*, 801–812.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual Retinal Progenitor Cells Display Extensive Heterogeneity of Gene Expression. PLoS ONE *3*, e1588.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Vaney, D.I., Peichl, L., and Boycott, B.B. (1988). Neurofibrillar long-range amacrine cells in mammalian retinae. Proc R Soc Lond B Biol Sci *235*, 203–219.

Vergara, M.N., and Canto-Soler, M.V. (2012). Rediscovering the chick embryo as a model to study retinal development. Neural Develop. *7*, 22.

Yamashita, T., Ono, K., Ohuchi, H., Yumoto, A., Gotoh, H., Tomonari, S., Sakai, K., Fujita, H., Imamoto, Y., Noji, S., et al. (2014). Evolution of mammalian Opn5 as a specialized UV-absorbing pigment by a single amino acid mutation. J. Biol. Chem. *289*, 3991–4000.

Young, R.W. (1985). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

6. Figures and legends

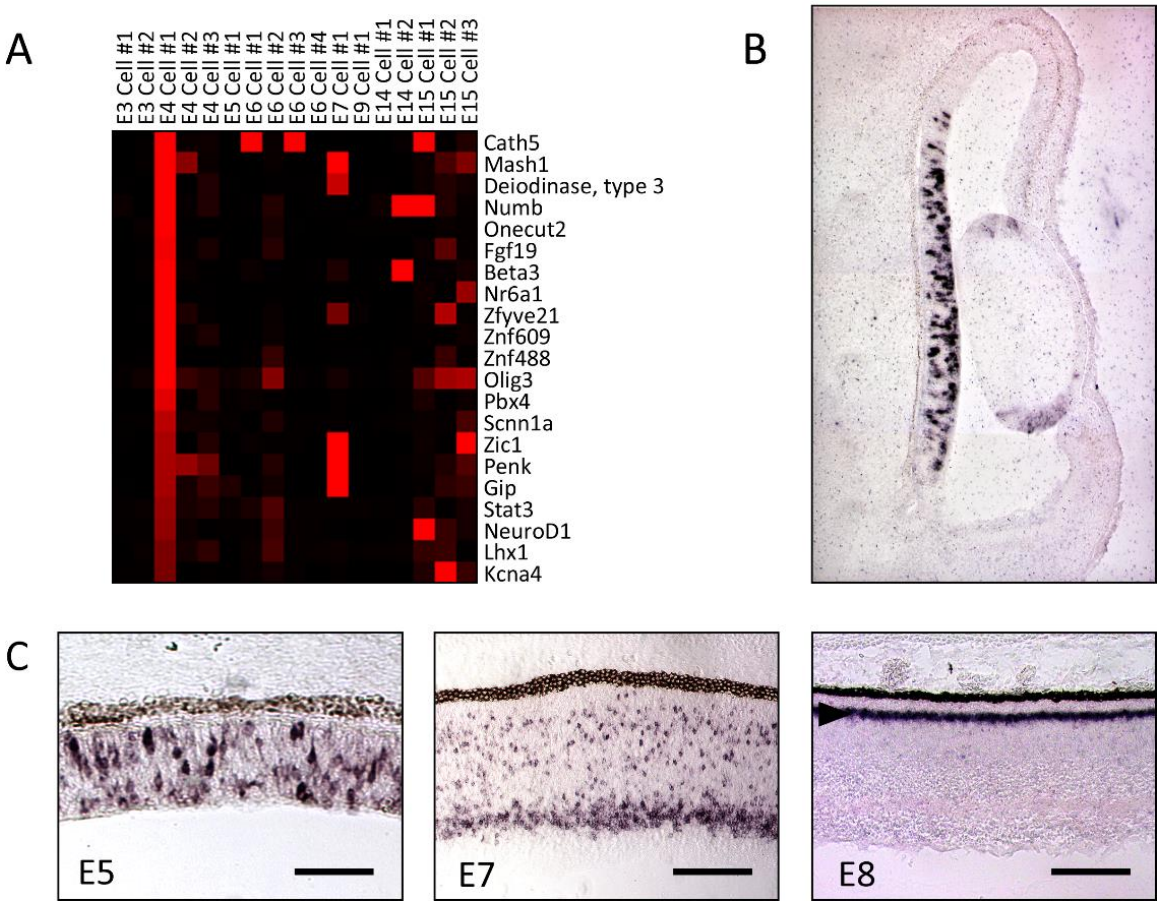

Figure 1. Fgf19 and other horizontal-specific genes

We were able to determine a horizontal cell present in our single-cell dataset (E4 Cell #1, A)using known horizontal cell marker Fgf19, the expression pattern of which is shown in composite through the early-developing retina (A), Normal expression patterns of horizontal cell marker Fgf19 is shown throughout retinogenesis (C). Although only one developing horizontal cell was identified in our single cell dataset, comparisons between it and the non-horizontal cells studied provided a great amount of information concerning horizontal cell genesis. Arrowhead indicates horizontal cells at the apical side of the INL. Scalebars represent 100 μm.

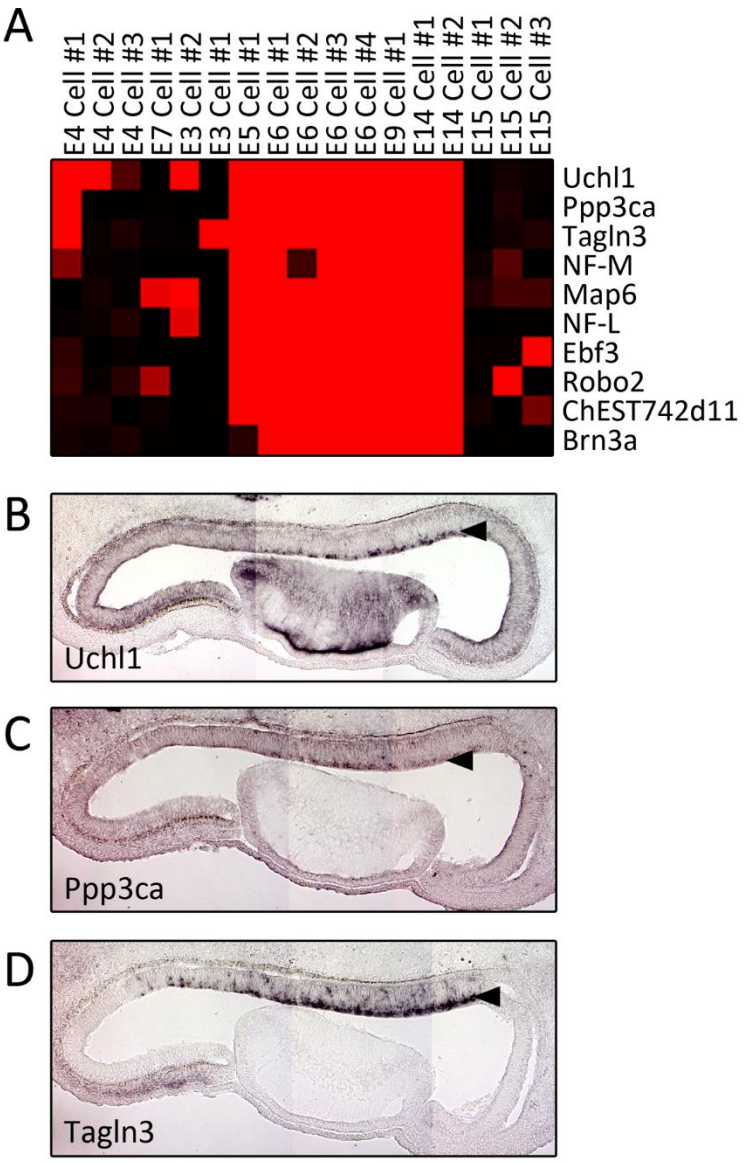

Figure 2. Analysis of retinal ganglion cells in the single-cell dataset

Retinal ganglion cells were identified within the single-cell dataset (A) through use of known markers. The specificity of markers such as Uchl1 (B), Ppp3ca (C), and Tagln3 (D) was then determined using ISH at E4. Arrowheads indicate the INBL.

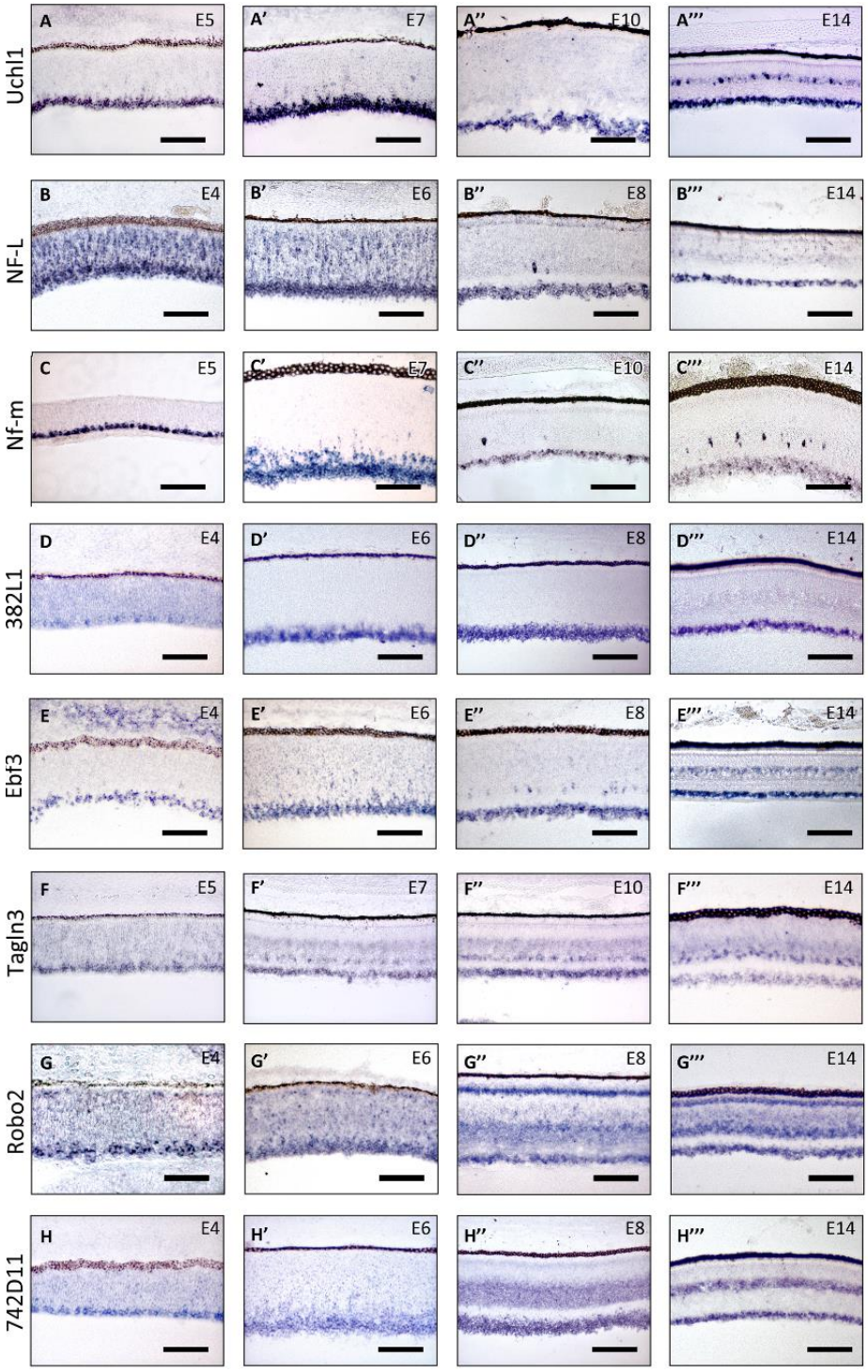

Figure 3. Genes with high correlation to RGC markers.

Other genes with high expression in RGCs were characterized using ISH at various timepoints throughout retinal development. Genes of interest included Uchl1 (A-A'''), NF-L (B-B'''), NF-M (C-C'''), 382L11 (D-D'''), Ebf3 (E-E'''), Tagln3 (F-F'''), Robo2 (G-G'''), and 742D11 (H-H'''). Scalebars represent 100 μm.

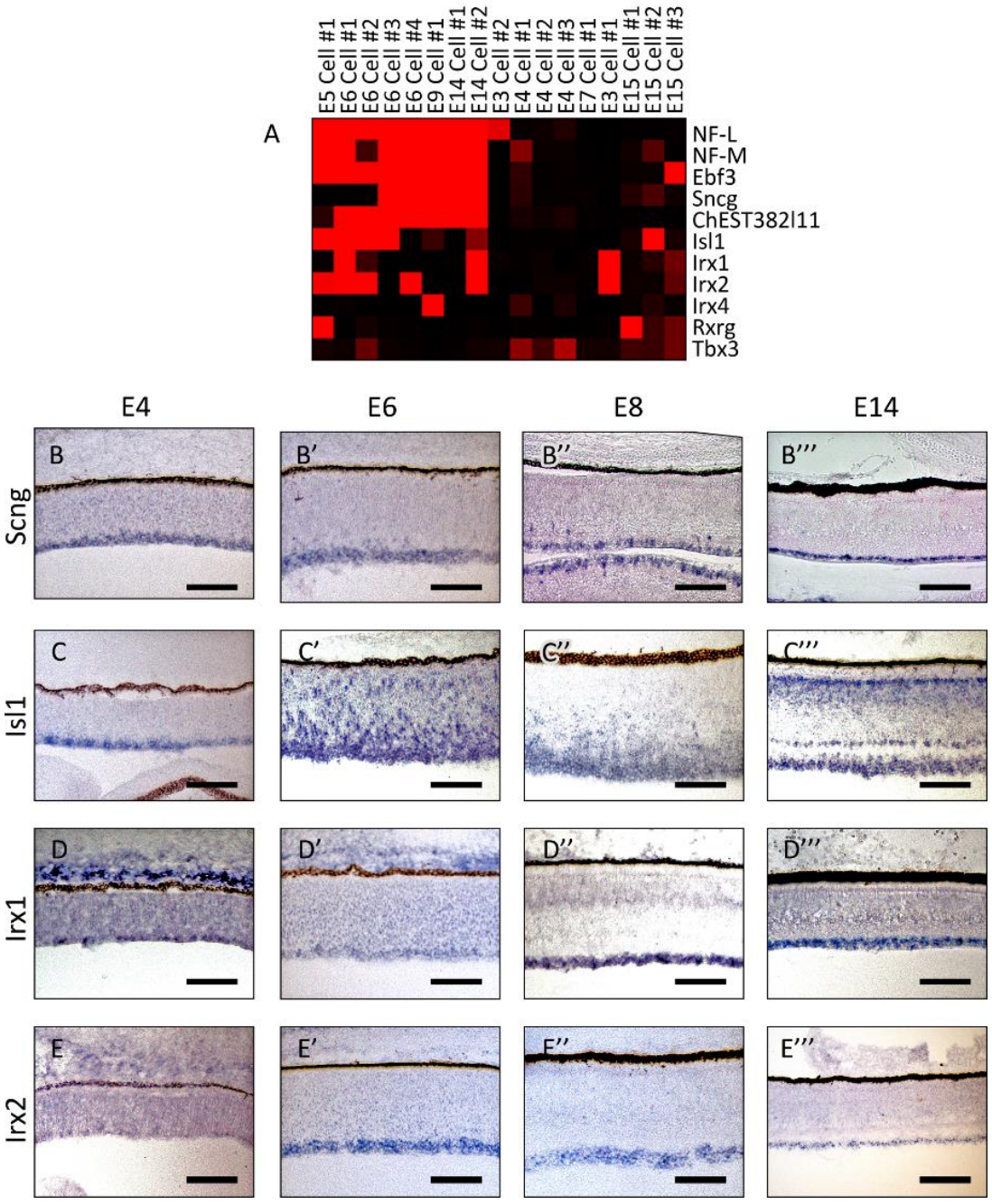

Figure 4. Genes observed in subsets of developing murine RGCs.

As a part of our main goal to determine the similarities and differences between the processes of vertebrate retinogenesis, we explored the expression patterns of genes identified in subsets of mouse RGCs (Trimarchi et al., 2007). (A) Genes identified in isolated mouse cells were also present in subsets of our chicken cells. The expression patterns for Scng (B-B'''), Isl1 (C-C'''), Irx1 (D-D'''), and Irx2 (E-E''') were confirmed as similar to those seen in mouse throughout retinogenesis using ISH. Scalebars represent 100 μm.

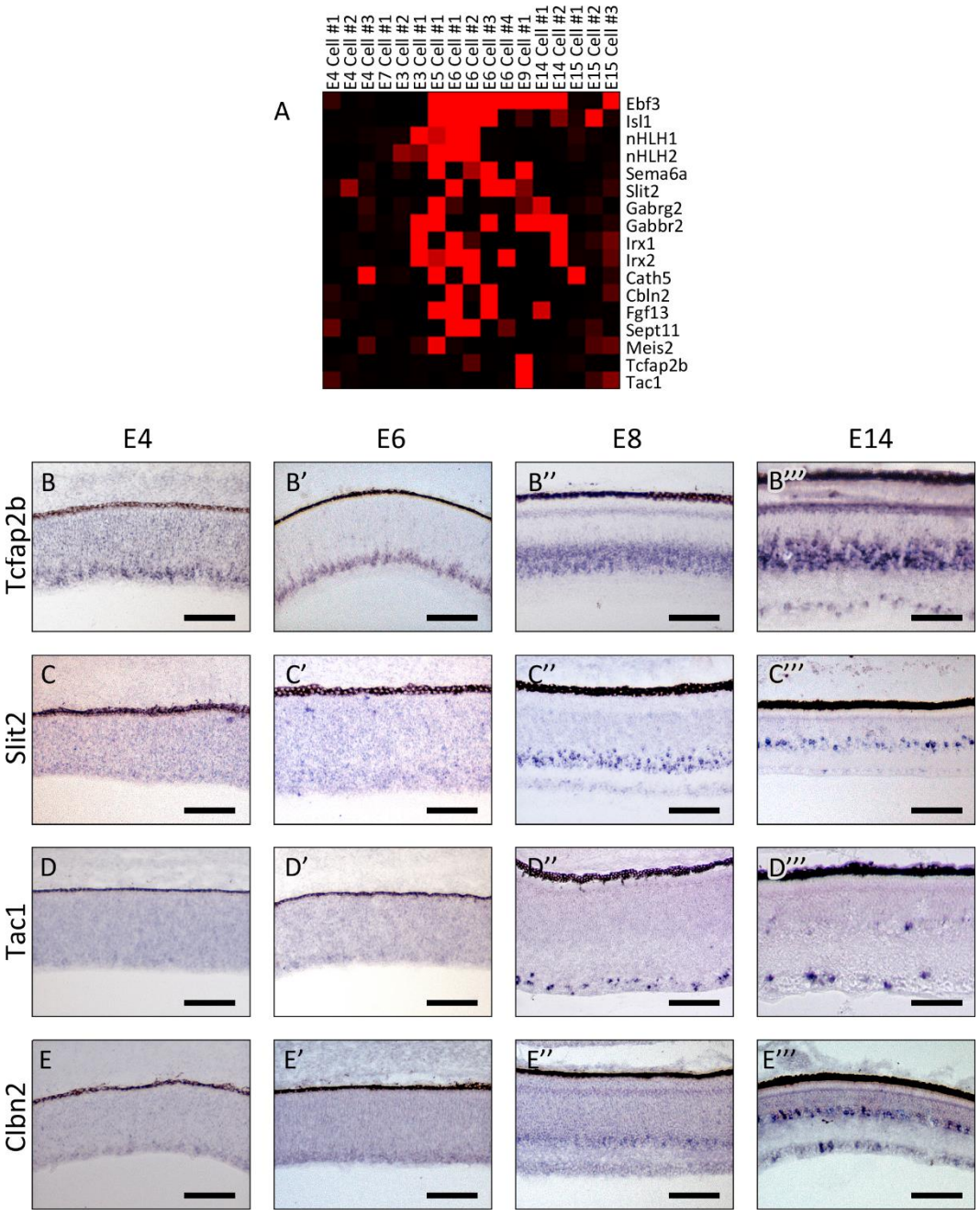

Figure 5. Genes present in single amacrine interneurons.

(A) After identifying multiple possible amacrine neurons in our dataset, including E9 Cell #1, and possibly the group of E5 and E6 cells showing highly heterogeneous expression of previously noted amacrine cell markers, ISH was performed to determine the expression patterns of genes in these diverse interneurons. Expression for Tcfap2b (B-B'''), Slit2 (C-C'''), Tac1 (D-D''') and Clbn2 (E-E''') are shown at various developmental timepoints, confirming their expression in amacrine populations. Scalebars represent 100 µm.

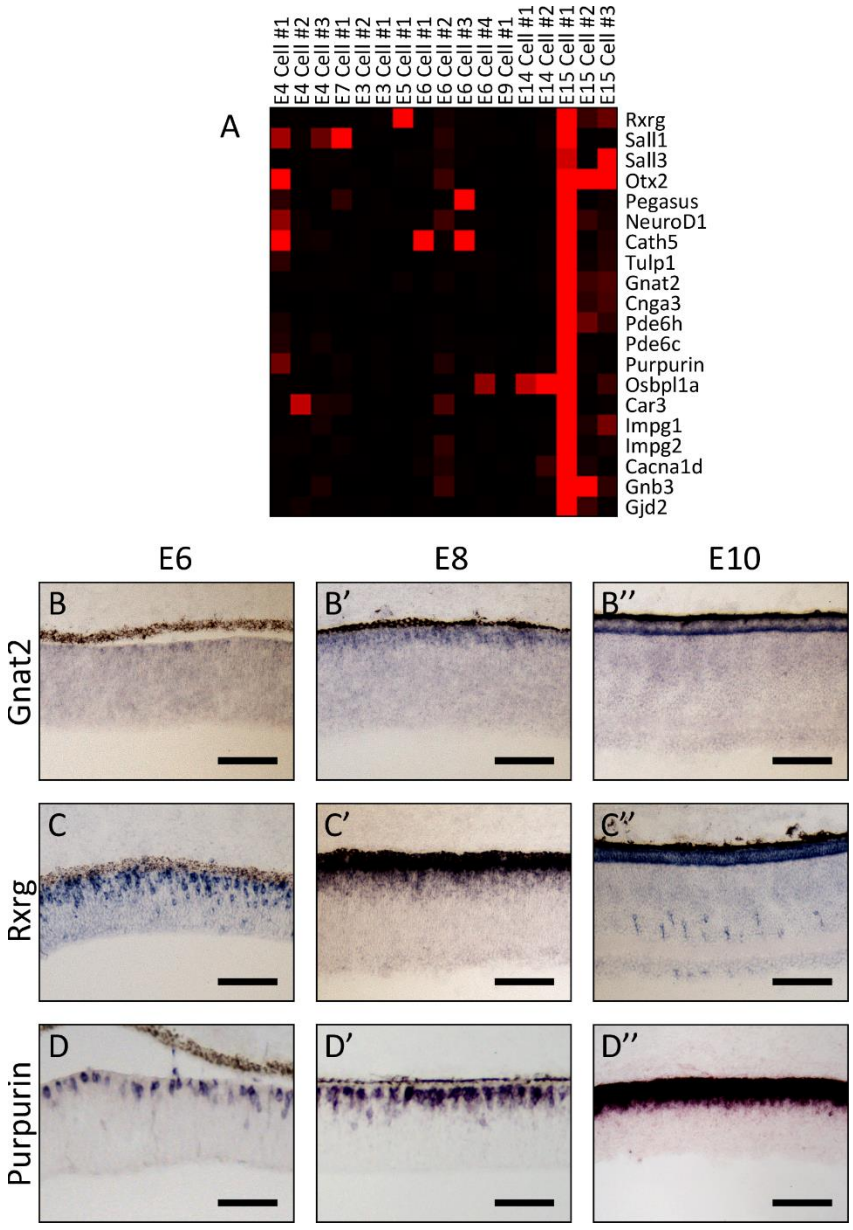

Figure 6. Markers of cone photoreceptors observed in chick retinal cells.

A single mature cone was identified in our collection single cell transcriptomes based on the presence of cone-specific transcription factors (A), and used to identify other markers of cone photoreceptors, including Gnat2 (B-B''), Rxrg (C-C''), Purpurin (D-D'') with expression patterns specific to cones. Scalebars represent 100 µm.

CHAPTER 5. GENERAL DISCUSSION

1. Introduction

The vertebrate retina is an instrument of great importance, not only due to its utility in the complex sensory experience of organisms, but also due to what it represents as an easily-isolated extension of the central nervous system, developing and functioning to sense and process input even before receiving input from other parts of the nervous system. Charles Darwin famously admitted that the evolution of the vertebrate eye with its "extreme perfection and complication" was "absurd in the highest possible degree," though not impossible given the known existence of transitional developmental states (Darwin and Wallace, 1998). Over the centuries, our increasing understanding of the phylogeny and ontogeny of the photosensitive tissue allowing the eye to function have only increased the wonder of those that study it. It is particularly interesting to realize that this complex sensory instrument forms over a relatively brief span from a common pool of retinal progenitors that, through a combination of cell-extrinsic and cell-autonomous signals, generate the prescribed amounts of neurons in a conserved order that then interconnect to make vision possible.

In this work, I have employed a novel technique for studying the transcriptomes of individual developing retinal cells to better determine the distinctive programs that drive those progenitor cells towards their eventual cell fates. Single-cell transcriptomics has proven to be an effective tool to determine the transcription factors present in subsets of developing cells that play critical roles in cellular differentiation (Chapter 2). The transcriptomes of individual cells have also provided insight into other genes expressed in progenitor cells at pivotal developmental timepoints, helping us to consider the roles of genes besides transcription factors in retinogenesis (Chapter 3). Lastly, while the information gleaned from developing mouse cells provides an excellent starting point to understand the processes leading to the fate determination and differentiation of retinal cells, my studies of transcriptomes from developing chick retinal cells (Chapter 4) is revealing the differences and similarities that distinguish the process of retinogenesis throughout evolutionary time.

2. Retinal transcriptomes reveal subsets of transcription factors present in Math5+ cells

2.1 The importance of Math5's role in the fate determination of early-born retinal cells

Math5 was immediately considered a gene of great interest in retinogenesis after its expression was found to be both downstream of and dependent on the dosage of Pax6, a homeodomain transcription factor critical to normal eye development (Brown et al., 1998; Grindley et al., 1995). Knockout studies in multiple model systems corroborated Math5's critical role as a requirement for establishing a retinal ganglion cell fate (Brown et al., 1998, 2001; Kay et al., 2001; Liu et al., 2001; Wang, 2001), and its overexpression was found to be sufficient to promote retinal ganglion cell fate in chicken and frog (Brown et al., 1998; Liu et al., 2001). Lineage studies revealed the complexities of Math5's role in early retinogenesis with the discovery that not only ganglion cells, but also cone photoreceptors, horizontal cells, and amacrine interneurons in the murine retina had a history of expressing Math5 – all early-born retinal cell types (Yang et al., 2003; Young, 1985).

While the exact details of Math5's influences on retinal cell fate decisions are perhaps unsurprisingly cryptic, it remains clear that its influence is critical to the generation of a normally-functioning retina from the onset of retinal cell differentiation. While studies have determined some genes acting downstream of Math5 as a result of expression changes after deletion of the transcription factor

(Mu et al., 2005), the direct relationships between Math5, many of these associated genes, and their effects on retinal cell differentiation remains unknown. Importantly, as in many whole-tissue studies, the heterogeneity between gene expression patterns within specific retinal cells is not taken into account with a clustering of the genes affected within the entire retina at various timepoints (Mu et al., 2005). Further insights into the role Math5 plays in early progenitor differentiation were provided when Math5+ and Math5- progenitors were sorted to determine differentially-expressed genes between the two populations (Gao et al., 2014). Here, it became clear that Math5+ cells had significantly lower growth factor expression than other cells in the same retina, while expressing higher levels of neurite guidance signals, indicating that Math5+ progenitors have exited the cell cycle and begun to differentiate (Gao et al., 2014).

Our single-cell studies have expanded on our understanding of the roles specific genes play in specific retinal progenitor cells by preserving the heterogeneity of a retinal progenitor in its own unique state of differentiation into a functionally distinctive cell at a given timepoint. In particular, comparing individual Math5+ retinal progenitor cells to Math5- cells in the developing retina can lead to a better understanding of the factors driving the fate determination and differentiation of specific early-generated retinal cell types. Our observations of these two populations of retinal progenitors led us to two transcription factors, Onecut1 (OC1) and its family member (OC2). Although the Onecut family of transcription factors was known to be expressed in the retina (Mu et al., 2001) they escaped notice in initial studies as downstream regulators of cell fate determination

using whole tissue approaches (Mu et al., 2005) but were later identified in specific subpopulations of retinal progenitors (Gao et al., 2014). Our extensive studies of the effects of these transcription factors is proof of principle for the utility of single-cell transcriptomics in determining effectors of cell fate in heterogeneously developing tissues such as the retina

2.2 Onecut transcription factors show a variety of responsibilities during retinogenesis

Single-cell transcriptomic analyses indicated that two Onecut family members, OC1 and OC2, were expressed in subsets of Math5+ retinal progenitors at an appropriate time to influence retinal cell fate decisions. Our initial surveys of Onecut1 and Onecut2 expression patterns confirmed that each transcription factor was highly expressed in the neuroblast layer during retinogenesis before resolving to the apical boundary of the inner nuclear layer during the postnatal maturation of the retina. With these expression patterns in mind, we obtained OC1 and OC2-knockout (KO) mice (Clotman et al., 2002) to determine whether developmental deficiencies existed in the absence of the Onecuts.

Morphological surveys of the adult retina showed vastly decreased horizontal cell populations in both OC1-KO and OC2-KO retinas. Wholemount staining of adult OC1-KO and OC2-KO retinas showed a decrease in horizontal cell populations of 65% and 80%, respectively. This phenotype was present from early retinogenesis, as qPCR of the horizontal cell marker Lhx1 was significantly

decreased in both E16.5 OC1-KO and OC2-KO mice. These results were all corroborated by conditional OC1 and OC2-null mice (Sapkota et al., 2014; Wu et al., 2013). Interestingly, overexpression of both OC1 and Ptf1a showed that these genes are both necessary and sufficient to generate horizontal cells, although neither gene alone will drive progenitors towards a specific fate (Wu et al., 2013).

My collaboration with the Cepko lab (Appendix 1) further elucidated the role of Onecuts with the determination that OC1 is not only necessary for the generation of a horizontal cell fate, but can also work in conjunction with a cis-regulatory module of the thyroid hormone receptor beta (CRM-Thrb) gene, which was shown to be present in retinal progenitors biased towards either a cone or horizontal cell fate (Emerson et al., 2013). Indeed, misexpression of OC1 alone is sufficient to drive retinal progenitors to express early markers of both horizontal cells and cone photoreceptors (Emerson et al., 2013). Additionally, my investigation of Thrb and the rod marker Nrl in OC1-KO retinas determined that loss of OC1 leads to decreased Thrb expression and increased expression of Nrl, a marker of rods, at E14.5 (Emerson et al., 2013). Given that OC1 loss leads to increased rod photoreceptor markers at the expense of cone markers, and that OC1 misexpression promotes either a horizontal interneuron or cone photoreceptor fate, it can be concluded that OC1 confers competence for either a horizontal interneuron or a cone photoreceptor fate, contingent on the coexpression or downstream expression of other factors.

While lethality in the full OC1 and OC2-KO mice made studies of the double-KO mice impossible in our lines, studies of conditional OC1-KO/OC2-KO further confirmed that the Onecut family works together to affect horizontal cell development. Whereas some horizontal cells are generated in either OC1 or OC2-KO retinas, retinas of conditional dKO mice generate no horizontal cells whatsoever, and also show defects in cone generation, with short-wave cones increasing at the expense of medium-wave cones (Sapkota et al., 2014). Another retinal population which may have been affected in our OC1-KO mice were the rare but important melanopsin (Opn4)-containing photosensitive ganglion cells, first noted by an upregulation of Opn4 in OC1-deficient mice on microarrays and confirmed by qPCR.

Additionally, other early-born retinal cell types, including starburst amacrines and retinal ganglion cells, appear affected in the dKO mice, further confirming the complexities of early retinal cell development (Sapkota et al., 2014). These results were further confirmed with a combination of OC1-conditional KO and OC2-KO mice, where additional surveys of horizontal cell markers were used to determine that while horizontal cells are formed in the early stages of retinogenesis, their populations are not maintained over time (Klimova et al., 2015). This finding is interesting given the understanding that OC1 biases progenitors towards a horizontal interneuron or cone cell fate. Although analysis of the development or maintenance of S-wave cones has not yet been performed, it may be that while OC1 is important for establishment of a progenitor's competency to generate a cone, it is required for the development of horizontal cells rather than the competence to generate them.

3. Explorations of genes expressed during retinogenesis with roles outside of direct transcription regulation

3.1 Plk3-null retinas develop normally *in vivo*

Among the other genes highly correlated with Math5 in our dataset of single cell transcriptomes was Polo-like kinase 3 (Plk3). Like its family members and their ancestral Drosophila *Polo,* this kinase has been shown to be capable of being an important regulator of the cell cycle (Zimmerman and Erikson, 2007a, 2007b). Indeed, while Plk3 is noted for its role as a transition from G1 to S phase (Zimmerman and Erikson, 2007a), and along with its family member, Plk2, is implicated in synaptogenesis and neurite maintenance (Pak and Sheng, 2003; Seeburg et al., 2005). Although possible roles for Plk3 exist in both generation and maturation of neurons, its expression patterns and functionality in the retina had not previously been established. Therefore, I sought to better understand any role Plk3 may play in retinal development. First, surveys of Plk3's expression throughout retinal development indicate patterns reminiscent of amacrine cell markers such as Tcfap2b, Tcfap2d, and Nhlh2. In the absence of Plk3 (Myer et al., 2011), surveys of gross retinal morphology in both developing and mature murine retinas are largely normal. However, transcriptomic analyses did indicate changes in some markers of subsets of retinal cells, including the marker of subsets of GABAergic amacrine

neurons Tac1, which was significantly decreased in Plk3-KO retinas, and retinitis pigmentosa 1 (Rp1), which was increased, indicating changes in photoreceptor populations. Despite the confirmation of these genes by multiple assays, other markers of these cell types were not affected. The implications of these gene expression changes on the functionality of the retina remains to be fully determined.

While these findings may seem difficult to interpret, the fact that there are such large and diverse populations of retinal cells could easily obscure a morphological phenotype, especially one affecting subsets of amacrine cells. Additionally, the main morphological phenotype that we originally observed in Plk3-KO mice was some amount of synaptic disorganization in the inner plexiform layer connecting the interneurons of the inner nuclear layer and retinal ganglion cells and amacrines of the ganglion cell layer. Multiple replicates were used to determine whether the disorganization was reproducible but scoring by an unbiased observer with experience in retinal organization led to narrowly insignificant differences between WT and Plk3-KO retinas (n=7 WT, n=9 KO; p=0.12). It's entirely true that deficiencies in generating small subsets of amacrine interneurons may cause subtle differences in gross retinal morphology.

It has been a challenge to discern a phenotype or lack thereof in the Plk3-KO mice. In many cases, I observed issues with retinal dissections, and between my past experiences with isolating retinas and the recurring issues with dissections only from the Plk3 colony, I found that I could blindly predict which retinas in a group were Plk3-deficient better than chance, as the Plk3-KO retinas do seem slightly

more fragile. Again, fellow graduate students were asked to attempt a dissection and noticed a similar phenomenon before I explained my concerns about the retinal texture. We have isolated and stained retinas from upwards of a dozen mice in attempts to determine the existence of a phenotype which still remains elusive, and our efforts are redoubled after each conference where we present antibody stains and casual observers reassure us that they see differences as well. Changes in my previous studies concerning Onecut loss were drastic and obvious. While any phenotype present in Plk3-KO mice will not be as dramatic as that resulting from Onecut transcription factor loss, consistent transcriptomics differences among timepoints, as well as the fact that similar morphological phenotypes have been discovered in at least some replicates at every timepoint we've observed (albeit without full penetrance), lead me to believe that Plk3 subtly affects some part of retinal development. It is not unprecedented for a gene expressed during retinogenesis to have either robust or subtle effects on retinal development that may not be immediately clear upon loss of the gene, especially *in vivo* (Kruger and Braun, 2002; Sarkisian and Siebzehnrubl, 2012). Therefore, it's entirely possible that we simply have not yet established the correct assay to understand those effects. Furthering our understanding of even subtle phenotypes when studying small but critical subpopulations of the retina including the diverse amacrine and ganglion cell populations is one small part of why studies to determine genetic markers of these subsets are so important.

3.2 The influence of model system on studies of neurogenesis

Individual cells in our dataset of murine retinal progenitors and neurons can exhibit present calls for around 9000 individual spots on the mouse microarray. These genes have wide ranges of functionalities, from transcription factors to neurite generation, targeting, or maintenance, to neurotransmitters and their receptors. Many of these genes are necessary for proper functioning of the neuron that expresses them, but none of these genes are expressed in full isolation. The complex interactions between genes and cells in the retina can influence the effects of loss of one particular gene in many ways.

In fact, some genes in the retina, such as bHLH transcription factors, can be used to partially recover the effects of loss of other related genes. Normally, loss of Math5 in the mouse consistently leads to loss of 80-90% of retinal ganglion cells (Brown et al., 2001; Wang, 2001). Loss of ganglion cells may be mitigated by other bHLH transcription factors, as seen when replacement of Math5 by bHLH factors NeuroD1 or Math3 could recover a respective 40% and 10% of retinal ganglion cells normally lost in the absence of Math5 (Mao et al., 2008). Recovery effects such as this are not universally attributable to all bHLH transcription factors, since misexpression of Ascl1 by the Math5 promoter showed no such recovery (Hufnagel et al., 2013). It's entirely possible that any responsibilities of Plk3 in the developing or mature murine retina could be similarly enacted by its family members,

especially Plk2 which has been implicated in many similar applications as Plk3 in the past (Pak and Sheng, 2003; Seeburg et al., 2005; Zimmerman and Erikson, 2007b). While this sort of redundancy is not unprecendented (Bassett et al., 2012), it is true that neither *in situ* hybridization nor microarrays indicated any significant upregulation of Plk family members including Plk2 at the mRNA level. Adding this fact to related studies suggesting that deficiencies in dendritic organization may be particularly difficult to discern *in vivo* due to the robustness of this process during neural development (Kruger and Braun, 2002; Sarkisian and Siebzehnrubl, 2012), more sensitive assays may be necessary to determine what phenotype, if any, exists in the absence of Plk3.

Additionally, the mouse model used to study a phenotype may have some effect on the findings of the study at hand. For instance, the Plk3-KO mouse obtained for our study did not show increased tumor formation at timepoints studied (Myer et al., 2011), leading the researchers to determine that Plk3 does not act as an oncogene in that system. While the Plk3-KO mouse in this study was generated by deletion of the promoter region and exons 1-6 (Myer et al., 2011), another knockout mouse was previously generated by removal of exons 1-8 (Yang et al., 2008). Although both mice lack the active sites conferring Plk3's kinase activity, the latter Plk3-KO mouse was found to exhibit increased tumorigenesis at advanced ages (Yang et al., 2008). The different findings of these two studies indicate the difficulty inherent in understanding the role of a single gene within a complex model system.

4. The future of single-cell isolation in the retina

4.1 Comparative transcriptomics

During retinogenesis, combinations of intrinsic and extrinsic factors influence a common pool of progenitor cells to differentiate and mature as any of six different kinds of neurons or one glial cell type, each of which plays a critical role in the retina's normal functioning (Masland, 2001; Young, 1985). While some of these cells are highly numerous and largely invariant, like the rod photoreceptors (Young, 1985), others are generated in much smaller numbers with much greater diversity, such as amacrine interneurons or retinal ganglion cells (Dräger and Olsen, 1981; Masland, 1988). Additionally, given that retinal cells are generated at stereotypically distinct and overlapping timepoints (Sidman, 1961; Young, 1985), progenitors destined for the same fate could be at different stages in fate determination or differentiation at the exact same timepoint in retinogenesis. Keeping these vast differences in representation and the heterogeneous timing of development in mind, single-cell analysis is one of the best ways to preserve the cell-autonomous programs that influence fate determination and lead to maturation as a specific retinal cell as each progenitor progresses throughout its own unique developmental timeline.

In the past, our lab has focused on the murine retina as a model system to determine the intrinsic signals that play a role in driving progenitor cells towards specific competencies or fates during retinogenesis. While we have gained indispensable insights into retinogenesis through single cell transcriptomics (Cherry et al., 2009; Trimarchi et al., 2007, 2008), the vast amount of information present in the transcriptome of over 200 mouse retinal cells can be difficult to parse as a collective source for understanding genetic pathways leading to specific cell fates. We sought to better understand the most developmentally significant signals in our transcriptomic dataset by expanding our frame of reference to include other vertebrate model systems. By including transcriptomes of developing cells from the chicken retina, we can better understand the genes that have maintained the same functionality by playing similar roles in neurogenesis throughout evolutionary time, and filter out less developmentally critical genes in the dataset, as well as species-specific transcriptomic peculiarities. We have isolated a number of chick retinal cells and identified horizontal cells, cone photoreceptors, amacrines, and ganglion cells in various stages of differentiation.

Even given the relatively small amount of chicken cells in comparison to our full mouse dataset, many similarities were noted between mouse and chicken retina cells. For instance, a single isolated progenitor initially drew attention due to its expression of the horizontal interneuron specific marker Fgf19 (Okamoto et al., 2009). At the same time, we were interested to note the same cell's expression of transcription factors more commonly seen in cones (Emerson et al., 2013). While our previous work had indicated that a network of genes including Otx2 and Onecut

family transcription factors could bias individual retinal progenitors toward either a cone or horizontal cell fate (Emerson et al., 2013), this mechanism had not yet been confirmed on the scale of a specific retinal progenitor cell Our initial surprise to find seemingly specific markers for two different cell types quickly gave way to confirmation of these previous studies as it became clear that this progenitor cell was poised on the cusp of determination between its fate as a horizontal interneuron or a cone photoreceptor. Beyond this interesting confirmation, commonalities were also determined between chicken and mouse progenitors as they progressed towards amacrine, cone, and ganglion cell fates.

These experiments show that comparative transcriptomics is a great starting point to better understand the critical intrinsic factors that act in conjunction to influence cell fate in retinal progenitors. I have also been involved in developing techniques to isolate retinal progenitors in developing zebrafish retinas as an extension of our understanding of comparative transcriptomics. As we develop computational strategies to compare and contrast the transcriptomes of retinal cells from various vertebrate species, we expect to gain more insights about the evolutionarily conserved genes and networks present to direct proper development and functionality of vertebrate retinas.

4.2 Combining transcriptomics with physiological studies

Another project that I have undertaken to better understand the transcriptomic signatures of individual retinal cells involves a combination of biological techniques. Although a better understanding of the development of retinal neurons is critical for applications such as generating individual retinal cells for regenerative therapies (Schmeer et al., 2012), that knowledge on its own cannot tie the development of each subtype of retinal cell to its eventual role within the mature sensory functionality of the retina. We have collaborated with electrophysiologists from Gregory Schwartz's lab at Northwestern University School of Medicine to characterize mature ganglion cells by functional subtype and stereotyped morphological structure before isolating their mRNA and analyzing their transcriptomes using RNAseq and microarrays (Flowchart for these techniques Figure 1). This combination of techniques will allow unprecendented insight into the factors that distinguish ganglion cells that differentially react to ON or OFF stimuli with transient or sustained bursts of activity, for example, or to determine the genes that predispose direction- and orientation-selective ganglion cells to their specific preferences. While this project is still at the beginning stages of analyzing preliminary data, the few dozen cells that we have already collected show great promise for the future of this collaboration.

5. Conclusions

During my time in the Trimarchi lab, I have employed a range of techniques to improve our knowledge of the development of the vertebrate retina in three separate model systems. The basis of all my experiments is single cell isolation, which allows access to the inner workings of individual retinal cells. In a common pool of retinal progenitors, each of which may be at any given stage of differentiation into any number of different retinal cells or subtypes during any specific point of retinogenesis, single-cell transcriptomics is a critical technique to preserve the heterogeneity that drives each retinal cell to its own unique fate. Using single-cell isolation to investigate genes present in subsets of retinal cells has allowed us to determine transcription factors critical for the development of large swaths of retinal cells, like the Onecuts, as well as genes with more subtle roles in retinogenesis, like Plk3. We hope to gain greater insights into the processes underlying retinogenesis through combining our transcriptomic datasets in novel ways, including better understanding the ways various vertebrate species compare to one another, as well as how transcriptomes can inform functional studies of mature retinal cells. These novel perspectives on retinal transcriptomes will undoubtedly aid in elucidating our current understanding of the formation and functionality of this amazingly complex tissue.

6. References

Bassett, E.A., Korol, A., Deschamps, P.A., Buettner, R., Wallace, V.A., Williams, T., and West-Mays, J.A. (2012). Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. Dev. Dyn. *241*, 814–829.

Brown, N.L., Kanekar, S., Vetter, M.L., Tucker, P.K., Gemza, D.L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Dev. Camb. Engl. *125*, 4821–4833.

Brown, N.L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Dev. Camb. Engl. *128*, 2497–2508.

Cherry, T.J., Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2009). Development and diversification of retinal amacrine interneurons at single cell resolution. Proc. Natl. Acad. Sci. *106*, 9495–9500.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. Dev. Camb. Engl. *129*, 1819–1828.

Darwin, C., and Wallace, J. (1998). The origin of species (Hertfordshire: Wordsworth Editions Ltd.).

Dräger, U.C., and Olsen, J.F. (1981). Ganglion cell distribution in the retina of the mouse. Invest. Ophthalmol. Vis. Sci. *20*, 285–293.

Emerson, M.M., Surzenko, N., Goetz, J.J., Trimarchi, J., and Cepko, C.L. (2013). Otx2 and Onecut1 Promote the Fates of Cone Photoreceptors and Horizontal Cells and Repress Rod Photoreceptors. Dev. Cell *26*, 59–72.

Gao, Z., Mao, C.A., Pan, P., Mu, X., and Klein, W.H. (2014). Transcriptome of Atoh7 retinal progenitor cells identifies new Atoh7-dependent regulatory genes for retinal ganglion cell formation. Dev Neurobiol.

Grindley, J.C., Davidson, D.R., and Hill, R.E. (1995). The role of Pax-6 in eye and nasal development. Development *121*, 1433–1442.

Hufnagel, R.B., Riesenberg, A.N., Quinn, M., Brzezinski, J.A. th, Glaser, T., and Brown, N.L. (2013). Heterochronic misexpression of Ascl1 in the Atoh7 retinal cell lineage blocks cell cycle exit. Mol Cell Neurosci *54*, 108–120.

Kay, J.N., Finger-Baier, K.C., Roeser, T., Staub, W., and Baier, H. (2001). Retinal Ganglion Cell Genesis Requires lakritz, a Zebrafish atonal Homolog. Neuron *30*, 725–736.

Klimova, L., Antosova, B., Kuzelova, A., Strnad, H., and Kozmik, Z. (2015). Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development. Dev. Biol. *402*, 48–60.

Kruger, M., and Braun, T. (2002). The Neuronal Basic Helix-Loop-Helix Transcription Factor NSCL-1 Is Dispensable for Normal Neuronal Development. Mol. Cell. Biol. *22*, 792–800.

Liu, W., Mo, Z., and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. Proc. Natl. Acad. Sci. U. S. A. *98*, 1649–1654.

Mao, C.A., Wang, S.W., Pan, P., and Klein, W.H. (2008). Rewiring the retinal ganglion cell gene regulatory network: Neurod1 promotes retinal ganglion cell fate in the absence of Math5. Development *135*, 3379–3388.

Masland, R. (1988). Amacrine cells. Trends Neurosci. *11*, 405–410.

Masland, R.H. (2001). Neuronal diversity in the retina. Curr Opin Neurobiol *11*, 431–436.

Mu, X., Zhao, S., Pershad, R., Hsieh, T.F., Scarpa, A., Wang, S.W., White, R.A., Beremand, P.D., Thomas, T.L., Gan, L., et al. (2001). Gene expression in the developing mouse retina by EST sequencing and microarray analysis. Nucleic Acids Res *29*, 4983–4993.

Mu, X., Fu, X., Sun, H., Beremand, P.D., Thomas, T.L., and Klein, W.H. (2005). A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. Dev Biol *280*, 467–481.

Myer, D.L., Robbins, S.B., Yin, M., Boivin, G.P., Liu, Y., Greis, K.D., Bahassi, E.M., and Stambrook, P.J. (2011). Absence of polo-like kinase 3 in mice stabilizes Cdc25A after DNA damage but is not sufficient to produce tumors. Mutat. Res. *714*, 1–10.

Okamoto, M., Bito, T., Noji, S., and Ohuchi, H. (2009). Subtype-specific expression of Fgf19 during horizontal cell development of the chicken retina. Gene Expr. Patterns *9*, 306–313.

Pak, D.T.S., and Sheng, M. (2003). Targeted protein degradation and synapse remodeling by an inducible protein kinase. Science *302*, 1368–1373.

Sapkota, D., Chintala, H., Wu, F., Fliesler, S.J., Hu, Z., and Mu, X. (2014). Onecut1 and Onecut2 redundantly regulate early retinal cell fates during development. Proc. Natl. Acad. Sci. *111*, E4086–E4095.

Sarkisian, M.R., and Siebzehnrubl, D. (2012). Abnormal levels of Gadd45alpha in developing neocortex impair neurite outgrowth. PloS One *7*, e44207.

Schmeer, C.W., Wohl, S.G., and Isenmann, S. (2012). Cell-replacement therapy and neural repair in the retina. Cell Tissue Res *349*, 363–374.

Seeburg, D.P., Pak, D., and Sheng, M. (2005). Polo-like kinases in the nervous system. Oncogene *24*, 292–298.

Sidman, R. (1961). Histogenesis of mouse retina studied with thymidine-H3. In The Structure of the Eye, G. Smelser, ed. (New York: Academic Press), pp. 487–506.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual Retinal Progenitor Cells Display Extensive Heterogeneity of Gene Expression. PLoS ONE *3*, e1588.

Wang, S.W. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev. *15*, 24–29.

Wu, F., Li, R., Umino, Y., Kaczynski, T.J., Sapkota, D., Li, S., Xiang, M., Fliesler, S.J., Sherry, D.M., Gannon, M., et al. (2013). Onecut1 Is Essential for Horizontal Cell Genesis and Retinal Integrity. J. Neurosci. *33*, 13053–13065.

Yang, Y., Bai, J., Shen, R., Brown, S.A.N., Komissarova, E., Huang, Y., Jiang, N., Alberts, G.F., Costa, M., Lu, L., et al. (2008). Polo-like Kinase 3 Functions as a Tumor Suppressor and Is a Negative Regulator of Hypoxia-Inducible Factor-1 under Hypoxic Conditions. Cancer Res. *68*, 4077–4085.

Yang, Z., Ding, K., Pan, L., Deng, M., and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. *264*, 240–254.

Young, R.W. (1985). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

Zimmerman, W.C., and Erikson, R.L. (2007a). Polo-like kinase 3 is required for entry into S phase. Proc. Natl. Acad. Sci. *104*, 1847–1852.

Zimmerman, W.C., and Erikson, R.L. (2007b). Finding Plk3. Cell Cycle Georget. Tex *6*, 1314–1318.

8. Figures and legends

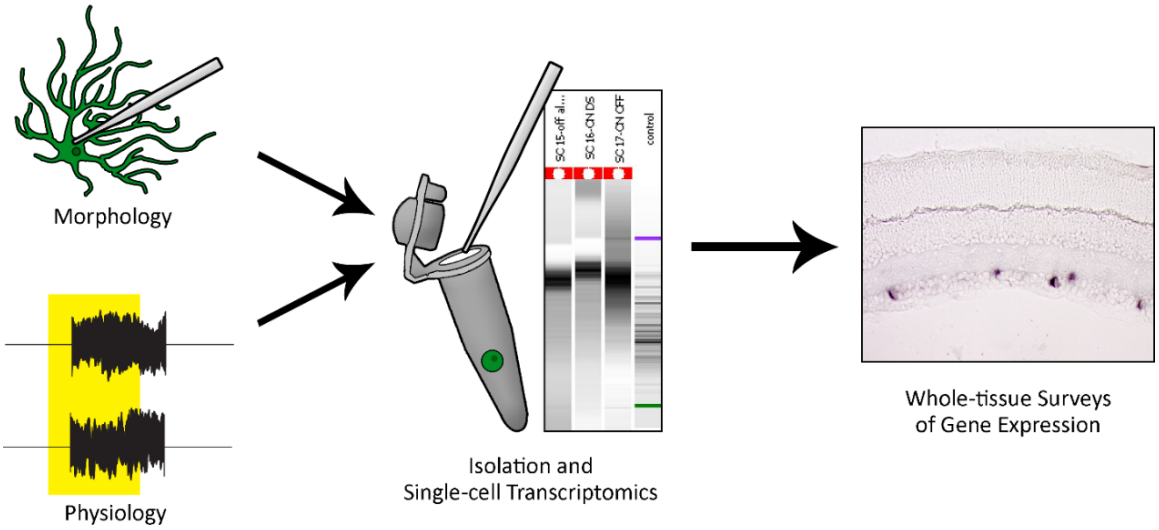

Figure 1. Flowchart of the collaboration between electrophysiological and transcriptomic methodologies

A flowchart outlining the steps of morphological, electrophysiological, and transcriptomic techniques involved in the Schwartz Lab collaboration.

APPENDIX. THE OTX2 AND ONECUT FACTORS PROMOTE CONE PHOTORECEPTOR AND HORIZONTAL CELL GENESIS OVER ROD PHOTORECEPTORS

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The author was responsible for experiments concerning Onecut1-KO mice.

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1. Abstract

Cone photoreceptors carry out phototransduction in daylight, thereby providing the critical first step in color vision. Despite their importance, little is known about the developmental mechanisms involved in their generation, particularly how they are determined relative to rod photoreceptors. We report here the identification of a cis-regulatory module (CRM) for the Thyroid Hormone Receptor Beta (Thrb) gene, an early cone marker. The ThrbCRM1 is active in

retinal progenitor cells biased to the production of cones and an interneuronal cell type, the horizontal cell (HC). We found that the ThrbCRM1 is regulated by Otx2 and members of the Onecut family of transcription factors. Onecut-1 is sufficient to induce cells with the earliest markers of cones and HCs. Conversely, interference with Onecut transcriptional activity leads to precocious rod development, suggesting that Onecut family members are critically important in defining cone versus rod fates.

2. Introduction

Vertebrate photoreceptor cells are a highly specialized class of cells that are responsible for capturing and processing light, initiating the first steps in vision (Rodieck, 1998). The two classes of photoreceptors are rod photoreceptors (rods) and cone photoreceptors (cones). Rods are able to respond to low light levels and use the photopigment, rhodopsin, to capture light. Cones mediate vision under daylight luminance conditions. They express a variable number of cone opsin photopigments, which allow for color vision. The importance of cones is underscored by the severe impairment of high acuity vision that accompanies their loss in the course of several human retinal diseases, such as retinitis pigmentosa and macular degeneration (Gehrs et al., 2006; Hartong et al., 2006). Development of therapeutic approaches for these diseases include the engraftment of photoreceptor cells generated by stem cells from various sources (Ong and da Cruz, 2012). The

limited numbers of cone photoreceptor-like cells generated in culture and their inefficient engraftment in the retina suggests that factors that can specifically increase the production of cones and endow them with *in vivo*-like properties for integration would be useful (see West et al., 2012, for example).

A number of investigations have sought to define the mechanisms of photoreceptor specification. The homeobox transcription factor, Otx2, is a critical component in this process as it is both expressed by all photoreceptors and is necessary for their genesis (Nishida et al., 2003). However, Otx2 is also necessary for the development of HCs and bipolar cells within the retina (Nishida et al., 2003; Koike et al., 2007). Its role in HCs is particularly intriguing as Otx2 has not been observed in identifiable HCs, suggesting that it may act in a very early step in their development (Emerson and Cepko, 2011). Recent work has identified a CRM for the Otx2 gene that is expressed in both early photoreceptors and early HCs, but not in bipolar cells, suggesting that there could be shared developmental mechanisms involved in the generation of photoreceptors and HCs (Emerson and Cepko, 2011). In addition to Otx2, the transmembrane receptor Notch has been implicated as a critical negative regulator of photoreceptor development (Jadhav et al., 2006; Yaron et al., 2006). However, the identity of other factors that cooperate with Otx2 and Notch to establish the photoreceptor cell fate are still unknown, though gain and loss of function studies of basic helix loop helix genes (bHLH) provide some evidence for their role in rod specification (e.g. Hatakeyama et al., 2001; Cherry et al., 2011).

The developmental relationship between cones and rods has been an area of great interest. These two cell types share a general function and specialized morphological features, as well as expression of genes that suggest there are common mechanisms involved in their development. However, there are key differences in a number of these characteristics that account for the unique identities of these cell types. Several studies have pointed to the Maf transcription factor, Neural Retina Leucine Zipper gene (Nrl), as the critical determinant of the rod versus cone photoreceptor fate (Mears et al., 2001; Daniele et al., 2005; Oh et al., 2007). According to recent models, a postmitotic photoreceptor precursor cell is generated during development that can give rise to either a cone or a rod. This common photoreceptor precursor has the cone cell fate as its default, but if it expresses Nrl, it will become a rod (Swaroop et al., 2010). Multiple lines of evidence support this hypothesis. In mice, which have rods as the dominant photoreceptor type, a knockout (KO) strain of Nrl had a large increase in photoreceptors with cone-like features and an absence of photoreceptors with rod-like features (Mears et al., 2001; Daniele et al., 2005). Conversely, misexpression of Nrl in all postmitotic photoreceptors leads to the absence of cone-like cells, suggesting that these cells have been transformed into rods (Oh et al., 2007). However, Nrl expression has been identified in developing cells with cone gene expression (Swaroop et al., 2010). In addition, it has been detected in the cytoplasm of human adult cones, suggesting that the expression of Nrl alone may not be sufficient in all contexts to drive rod genetic programs (Swain et al., 2001). While several studies provide evidence to support the existence of a common precursor, the interpretation of these

experiments has relied largely on changes in gene expression of mature photoreceptors. Thus, it is unclear whether Nrl regulates the fate choice of rods vs cones at the time of their genesis, or whether it regulates later gene expression i.e. Nrl may be responsible for the repression of cone genes and the expression of rod genes, normally after an upstream determination event. Furthermore, the genetic programs that drive early cone gene expression are unknown.

Retinal cell types are generated from RPCs in overlapping windows of developmental time, with retinal ganglion cells, cones, and HCs born almost exclusively in the embryonic retina, and bipolar cells and Mueller glia born mainly in the postnatal period in mice and rats. Rod and amacrine cell genesis span both the embryonic and postnatal periods (Sidman, 1961; Carter-Dawson and LaVail, 1979; Young, 1985). Early studies of lineage in the rodent retina using retroviral labeling demonstrated that many RPCs were multipotent and were able to produce overlapping combinations of cell types (Turner et al., 1990). However, recent work has suggested that, at least in some cases, a terminal division of a specific type of RPC generates particular daughter cell types. We recently reported that in the mouse, we could direct retroviral infection to RPCs that expressed Olig2, a bHLH gene (Hafler et al., 2012). Infection of embryonic day 13.5 (E13.5) revealed that the Olig2+ RPCs produced only cones and HCs (referred to hereafter as an RPC[CH]). In the postnatal period, the Olig2+ RPC population generated almost exclusively rods and amacrine cells (Hafler et al., 2012). Moreover, work in both chicken and fish have demonstrated the existence of a RPC that makes only HCs (Godinho et al., 2007; Rompani and Cepko, 2008). A further restriction was noted in chick, with a

terminal division producing homotypic paris of HC type 1 or type 3 cells, but not of HC type 2 (Rompani and Cepko, 2008). The molecular identity of the pathways that establish these restricted progenitor states are unknown.

In order to better understand the developmental origins of cones, we have indentified a CRM for the Thrb gene, one of the earliest and most specific cone genes known to date (Ng et al., 2001, 2009). The evidence presented here suggests that this element is active specifically in RPCs that generate HCs and cones and is co-regulated by Otx2 and Onecut factors. These factors are co-expressed in a subset of embryonic RPCs and are also co-expressed with Olig2. Misexpression of Onecut1 in the postnatal period is sufficient to induce the expression of early markers of both cones and HCs. Conversely, loss-of-function studies support a role for Onecut factors in promoting the cone fate at the expense of the rod fate and suggest that these factors act genetically upstream of Nrl. These studies identify key molecular components that establish the RPC[CH] state and identify Onecut factors as critical determinants of cone versus rod identity.

3. Materials and methods

Bioinformatics

Candidate CRMs were identified using the ECR browser (http://ecrbrowser.dcode.org/) and UCSC blat server (http://genome.ucsc.edu/cgi-bin/hgBlat) and the homologous sequence in each species were garnered from these programs or by manual inspection of the Thrb region for the conserved motif. Binding site logos for Onecut1 and Otx2 are from Transfac (Matys et al., 2006).

DNA electroporations

Ex vivo electroporations and culture were carried out as described (Emerson and Cepko, 2011). Chicken spinal cord electroporations were carried out on stage 18 embryos using DNA concentrations of 0.5μg/μl for CAG constructs and 1.0μg/μl for ThrbCRM1-AU1. Fast green (2μg/μl final concentration) was included to visualize DNA injections into the central canal of the spinal cord at the thoracic level. A sharp tungsten electrode was inserted into the embryo on the left side of the neural tube and a gold plated positive electrode was placed on the right side. Three pulses of 10V, 50ms each, 950ms apart were applied. Eggs were sealed with tape and incubated for 1 additional day before harvest. Processing was carried out identically to that for the retina for immunohistochemistry. *In vivo* electroporations of mouse P0 CD-1 pups were as described (Cherry et al., 2011). P0-P30 electroporations used a floxed version of CAG-OC1 described in the supplemental methods.

Immunohistochemistry

Unless described below, antibody sources, concentrations and conditions were as described (Emerson and Cepko, 2011). Additonal primary antibodies and dilutions were as follows: Goat anti-Otx2 (R&D Systems, AF1979, 1:500), Rabbit anti-Olig2 (Millipore, ab9610, 1:1,000), Rabbit anti-OC1 (Santa Cruz Biotechnology, sc-13050, 1:100), Sheep anti-OC2 (R&D Systems, AF6294, 1:500), Rabbit anti-RXRγ (Santa Cruz Biotechnology, sc-555, 1:100), Mouse anti-βgalactosidase (Developmental Studies Hybridoma Bank, 40-1a-conc., 1:20), and Rabbit anti-Nr2e3 (gift from Jeremy Nathans, JH707, 1:500). The Rabbit anti-Otx2 antibody was used in combination with antibodies to OC2, while the Goat anti-Otx2 antibody was used in combination with antibodies to OC1 and Olig2.

EdU labeling was performed by I.P. injecting pregnant dams with 150 μl of 10mg/ml EdU resuspended in 1XPBS. EdU detection was performed with a Click-iT EdU Alexa Fluor 647 Imaging kit (C10340, Invitrogen) according to the manufacturer's instructions.

Section RNA *in situ* hybridization

See supplemental methods for RNA probes generation. Methodology for mThrb and cThrb RNA detection by AP development was as described (Supplemental Methods of Trimarchi et al., 2007) and cThrb RNA detection was the same except that anti-digoxigenin (DIG) coupled to POD instead of AP was used and the slides were washed several times in TNT (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl,

0.05% Tween-20) following the α-DIG POD incubation and MABT washes. Slides were processed for tyramide amplification using Cy3-tyramide (Perkin Elmer) at 1:100. After 20 minutes of incubation in developing solution, slides were washed three times in TNT and then washed in PBT and processed in this study's standard antibody processing procedure for GFP. Methodology for L-Maf RNA hybridization was as described in (Trimarchi et al., 2007) except that the last fixation step was not performed after AP staining and the sections were processed for βgal immunofluorescent detection according to the procedure used throughout this study.

Dissociated cell *in situ* hyrbridization

After retinal explants were cultured *ex vivo* using our standard conditions for 8 hours, they were digested into a single cell suspension with papain and cells were incubated on Poly-d-lysine coated slides for 30 minutes at room temperature in DMEM plus 10% Fetal calf serum and fixed with 4% paraformaldehyde for 10 minutes. The protocol for DISH described in Trimarchi et al. 2007 was followed with the following modifications: no methanol dehydration was performed, incubation time with α-DIG-POD was 1.5 hours at room temperature and post-development fixation was not performed. Slides were then processed for immunohistochemistry to detect βgal and GFP and nuclei were stained with DAPI. Three electroporated retinas were analyzed.

Chromatin immunoprecipitations and analysis

Chromatin preparation and immunoprecipitation was performed with the EZ Chip kit (Millipore, 17-371) according to the manufacturer's instructions. Seven E5 retinas dissected away from retinal pigmented epithelium, lens and vitreous were used for each biological sample – this was done in triplicate. Chromatin was prepared in a Bioruptor (Diagenode) using 3 pulses for 5 minutes each (total time of actual sonication was 7.5 minutes. 1/12 of the sample was used for each immunoprecipitation. 10 micrograms of each antibody (Anti-Otx2, ab21990, Abcam; Anti-OC1, sc-13050, Santa Cruz Biotechnology; Normal Rabbit IgG, sc-2027, Santa Cruz Biotechnology) were used in each immunoprecipitation. See Supplemental Methods for primer sequences and qPCR details.

Imaging and image processing

Confocal imaging was as described (Emerson and Cepko, 2011), and in addition some images were taken with a Leica SP2 upright confocal. Image processing was performed with Imaris software. Images were uniformly adjusted within an image and between samples in a group. Several images (Figure 2K-R, Figure 3Q-U, Figure 5Q-T) use maximum intensity projections for illustrating colocalization for presentation purposes, but colocalization was verified by single z-plane analysis for all such images. All quantitation was done as in (Emerson and Cepko, 2011). All retinal section images are oriented with the sclerad side up.

Animals

All methods used in animal studies were approved by the Institutional Animal Care and Use Committee at Harvard University.

4. Results

Early cone photoreceptor gene expression is not regulated by Nrl

The prevailing model of photoreceptor genesis posits that a postmitotic cell is specified to become a photoreceptor by expression of Otx2, along with other unidentified factors (Figure 1A). Expression of the Maf family transcription Nrl in these cells is thought to suppress the program that results in the genesis of cones, while promoting the rod fate (Swaroop et al., 2010). The foundation for this model is the observation that adult photoreceptors in Nrl knockout (KO) mice lack rod-specific markers and upregulate cone-specific ones (Mears et al., 2001; Daniele et al., 2005; Hsiau et al., 2007). However, it is unclear whether these photoreceptors are completely or partially transformed into cones and when this change occurs developmentally. In order to investigate these questions, we examined the expression of Thrb and RXRγ genes in the developing retina of Nrl KO mice. These two genes have been used to positively identify newborn cones and are thought to be absent in rods (Mori et al., 2001; Ng et al., 2001, 2009; Roberts et al., 2005). If Nrl is indeed the most upstream factor driving the decision of a photoreceptor precursor to become a rod, then these early cone genes should be upregulated in Nrl

KO photoreceptors during the period when rods are normally generated. In contrast to this prediction, qPCR analysis revealed that both Thrb and RXRγ were unchanged in P0 Nrl KO retinas; a time when a large number of rods are normally being produced, and when approximately 50% of rods have already been produced from genesis in the embryonic period (Figure 1B) (Carter-Dawson and LaVail, 1979). This is in agreement with a previous study which identified no upregulation of RXRγ at P2 in the Nrl KO mouse (Yoshida et al., 2004). However, this study did detect a significant increase in RXRγ expression at P10 suggesting that eventually RXRγ is dysregulated by loss of Nrl (Yoshida et al., 2004). In contrast, a dramatic reduction in the expression of the Nrl target gene, Nr2e3 (also known as Photoreceptor-Specific Nuclear Receptor), was observed in the Nrl KO, confirming that this is normally a time of active transcriptional regulation by Nrl (Figure 1B). These data suggest that additional factors regulate early cone gene expression independently of Nrl function.

Identification of Thrb cis-regulatory modules

To identify gene regulatory networks (GRNs) that are involved in cone genesis we sought to identify CRMs that were specifically active in the development of cones. Identification of CRMs responsible for Thrb expression were targeted as Thrb is thought to be the earliest and most specific marker of cones (Ng et al., 2009). Phylogenetically conserved genomic elements in proximity to the Thrb locus were

identified and tested for their ability to drive reporter gene expression in a Thrb-like pattern (Figure 2A-C, S1A). Reporter constructs were tested in the chicken retina by *ex vivo* electroporation because of the large number of cones in the chick, as well as our previous work showing that it was a robust system for CRM identification in the retina (Emerson and Cepko, 2011). Two elements, ThrbCRM1 and ThrbCRM2, were found to have positive activity in the retina compared to a control enhancer when introduced at embryonic day 5 (E5), though they showed differences in expression (Figure 2D-G, 2D'-G'). ThrbCRM1 was active in a subset of electroporated cells 20 hours after introduction of the reporter plasmid, while ThrbCRM2 was active in only a very small number of cells at this time (data not shown). The majority of the ThrbCRM1^{+} cells were located just vitreal to the scleral surface in the developing photoreceptor layer. At 2 days after DNA introduction, the retinas electroporated with a control mGnat1CRM plasmid that would not be expected to be active had no EGFP expression (Figure 2D,D'). In contrast, ThrbCRM1 drove reporter expression in newborn photoreceptors, as marked by Visinin staining, as well as in other cells just vitread to the scleral surface (Figure 2E,E') (Fischer et al., 2008). In addition, it labeled a number of cells just on the scleral side of the vitreal surface. In contrast to the 20 hour timepoint, by 2 days post electroporation, ThrbCRM2 was now active in Visinin-positive cells, but did not appear to have activity in the other two populations marked by ThrbCRM1 reporter activity (Figure 2F,F'). Previously, a CRM referred to as a Thrb intron control region (ThrbICR) was isolated, which was described as directing expression to postmitotic cones as well as retinal ganglion cells using transgenic mice (Jones et al., 2007). In order to test if this element could

have activity similar to that of ThrbCRM1 or ThrbCRM2, the mouse ThrbICR element was cloned into the same reporter vector and introduced into chicken retinas. Like ThrbCRM1, the ICR was expressed in Visinin-positive photoreceptors as well as a number of other cells (Figure 2G,G'). As both ThrbCRM1 and ThrbICR had activity shortly after electroporation, it seemed possible that these two elements represented partially redundant CRMs that captured the same GRN. In order to determine if this was a possibility, co-electroportation of two plasmids encoding the two elements upstream of unique reporters were introduced into the chicken retina. A large number of cells co-expressed the two reporters, suggesting that these two elements could share a GRN (data not shown).

Sequence elements of ThrbCRM1 and restricted activity to the period of cone genesis

One model for the expression of ThrbCRM1-GFP in a subset of RPCs, HCs, and photoreceptor cells is that there is a direct relationship among these three cell types. For instance, it could be that ThrbCRM1-GFP is active in RPCs that generate cones and HCs, and no other cell type. Intriguingly, we recently discovered such a RPC in the mouse. A knock-in allele of olig2 that places the avian viral receptor, TVA, under the regulatory sequences of the olig2 locus was used (Hafler et al., 2012). Infection with EnvA-coated retroviruses, which require TVA for viral entry, infected only those cells that have TVA (Olig2-positive cells). Because the type of

retrovirus used in this experiment was a gammaretrovirus, which requires the nuclear envelope breakdown that occurs during mitosis in order to integrate into the genome, only cells derived from an Olig2-positive RPC were labeled, i.e. postmitotic cells that expressed TVA would not host viral infection (Roe et al., 1993). When infection was carried out *in vivo* at E13.5, Olig2-derived clones were almost exclusively composed of cones, HCs, or both cell types identifying this RPC type as the RPC[CH]. During the postnatal period, Olig2-positive progenitors were still present, but did not produce cones or HCs, in accord with the lack of cone and HC genesis in the postnatal period. Instead, they produced rods and amacrine cells.

In order to test whether the ThrbCRM1-positve RPCs might be functionally equivalent to the Olig2-positive RPCs of the embryonic mouse retina, we wanted to use a TVA/EnvA-like strategy in the chicken retina. Because chicken cells express the TVA receptor, another viral receptor not normally expressed by chicken cells was required. The murine ecotropic receptor, CAT1, from Moloney murine leukemia virus (MMLV) was chosen because of its restricted expression to murine species, its demonstrated ability to confer susceptibility to viruses carrying the ecotropic, MMLV gp70 envelope protein, and its proven ability in the developing chicken nervous system to infect electroporated cells expressing a CAT1 plasmid (Albritton et al., 1989; Gotoh et al., 2011). Three enhancer/promoter regions were placed upstream of the CAT1 coding sequence – a basal promoter with no enhancer sequences as a negative control, the ThrbCRM1 enhancer in conjunction with the same basal promoter, or the CAG enhancer/promoter construct, which should provide ubiquitous expression as a positive control. Constructs were electroporated

into E5 chicken retinas with a co-electroporation control, and were infected 6 hours later with MMLV gp70 coated retroviruses encoding a GFP reporter. Retinas were assayed for GFP expression 36 hours after infection.

In retinas electroporated with the basal promoter-CAT1, very few GFP-positive cells (2 cells out of 3 retinas) were observed, supporting both the idea that retroviruses with MMLV gp70 are unable to infect cells without the ecotropic receptor, and that the basal promoter by itself has a very low level of background expression (Figure 2H,H'). In contrast, retinas electroporated with CAG-CAT1 had large patches of GFP-positive infected cells in electroporated areas (Figure 2I,I'). Interestingly, examination of retinas electroporated with the ThrbCRM1-CAT1 receptor construct revealed that a much smaller number of GFP-positive cells were infected with virus compared to the CAG-CAT1 condition (Figure 2J,J'). Unlike the GFP-positive cells observed with CAG-CAT1, the ThrbCRM1-derived cells were not found throughout the radial dimension of the retina, but were found primarily in the developing photoreceptor layer, or just scleral to the vitreal surface, where developing HCs are often found. In order to determine if ThrbCRM1-derived cells preferentially assumed the photoreceptor or HC fate, GFP-positive cells were co-localized with specific markers of these cells (Visinin for photoreceptors and Lim1 for HCs)(Figure 2K-R). A significant enrichment for both photoreceptors (~10 fold) and HCs (~3 fold) were observed from CAT1 expression driven by the ThrbCRM1 element compared to the CAG element (Figure2S). This experiment demonstrates that the ThrbCRM1-positive RPC is biased to the production of these two cell types and likely corresponds to a RPC[CH].

Otx2, Olig2 and Onecut family members are co-expressed in RPCs

In order to determine what transcription factors might regulate the activity of ThrbCRM1, we examined the sequence of the 40 bp critical region of ThrbCRM1. We found that analysis of the sequence by Transfac suggested that the 8bp sequence shared with ThrbICR could be regulated by the Onecut family of transcription factors. In addition to their sequence binding specificity, Onecut1 (OC1) and Onecut2 (OC2) have recently been shown to have expression in embryonic RPCs during the period of cone and HC genesis (Wu et al., 2012). In late embryonic and postnatal time points, these genes are not expressed in RPCs (Wu et al., 2012). This restricted expression pattern makes these factors excellent candidates for regulating Thrb, and possibly other genes, in a RPC[CH]. At later time points and into the postnatal period, the Onecut (OC) genes are expressed in HCs, at a level much higher than that seen in RPCs (Wu et al., 2012). Furthermore, the sequence on either side of the predicted OC binding site closely matched a predicted Otx2 binding site. As Otx2 is a gene necessary for both HC and photoreceptor genesis and is expressed in embryonic RPCs, it too was an excellent candidate for regulating the ThrbCRM1 element (Nishida et al., 2003; Sato et al., 2007; Emerson and Cepko, 2011; Muranishi et al., 2011).

If Otx2 and Onecut factors collaborate to regulate the ThrbCRM1 element in RPC[CH]s, it would be expected that they would be expressed in the same RPCs.

Examination of RPCs (as identified by EdU labeling) in the embryonic mouse revealed that a large number of Otx2-expressing RPCs also expressed OC1 and OC2 (Figure 3A-P). The overlap of OC1 and OC2 with Otx2 in the neural retina was not because of cross-reactivity with the primary or secondary antibodies because there were clearly Otx2-positive cells in the RPE that were not positive for Onecuts, and there were Onecut positive cells in the GCL layer that were not positive for Otx2. In order to determine if Onecut factors were present in the RPC[CH], Olig2-positive RPCs were examined and OC2 was found to be expressed in these cells (OC1 and Olig2 co-localization could not be tested because the antibodies were from the same host)(Figure 3Q-U). In addition, examination of the chicken retina revealed Olig2/Otx2 double-positive cells, and Otx2/OC1 double-positive cells (data not shown). Some of the Otx2/OC1 double-positive cells were also positive for Visinin, suggesting that OC1 is maintained in postmitotic cones for some amount of time in agreement with a previous study in the mouse (data not shown)(Muranishi et al., 2010).

Onecut1 is sufficient to induce the ThrbCRM1 reporter activity in the postnatal retina in an Otx2-dependent manner

OC1 and OC2 are both expressed in mouse RPCs at E12.5 and E14.5, but cease to be in RPCs after E16.5 (Wu et al., 2012). We hypothesized that the expression of OC1 and OC2 may be the molecular correlates of the competence window that restricts the production of cones and HCs to the embryonic retina. While OC1 and OC2 are no longer present in RPCs of the postnatal retina, Otx2 is

still expressed in a large number of postnatally produced cells. To investigate whether an OC/Otx2 complex could regulate the ThrbCRM1 element, mouse OC1 was introduced into RPCs of the postnatal mouse retina with a ThrbCRM1-PLAP reporter. Robust PLAP expression was induced by OC1 expression, but not by mOtx2, mNeuroD1, or mNeuroD6 (data not shown). The lack of activation by mOtx2 given its ability to induce ThrbCRM1 reporter in other contexts (see Figure 4), suggests that mOtx2 requires a partner in order to initiate transcription, presumably an OC family member.

Otx2 induction of ThrbCRM1 reporter and Thrb mRNA in the spinal cord

The fact that mOC1 was sufficient to induce the ThrbCRM1 reporter in the high Otx2 expression environment of the P0 mouse retina prompted the converse question - whether mOtx2 is sufficient for the induction of the reporter in a OC-positive environment? The spinal cord has recently been shown to express all three Onecut family members, but has not been reported to express Otx2 (Francius and Clotman, 2010).

Electorporations of the chick spinal cord were carried out to further investigate the need for OC family members and Otx2 for ThrbCRM1 expression. When chicken E3 spinal cords were electroporated with the ThrbCRM1 reporter, no reporter induction was detected, suggesting that critical regulatory factors were absent (Figure 4A-D). It was also confirmed that spinal cord expression of Otx2

could not be detected by immunohistochemistry, while OC1 was confirmed to be present (Figure 4B,C). Electroporation of CAG-mOC1 with the ThrbCRM1 reporter was also ineffective at inducing reporter expression (Figure 4E-H). Given the robust expression from ThrbCRM1 reporters in the mouse P0 retina coelectroporated with the mOC1 construct (data not shown), this clearly shows the context-dependent nature of Onecut activity, which is likely linked to Otx2 expression. In support of this hypothesis, when mOtx2 was coelectroporated with the reporter into the spinal cord, induction of the reporter was observed (Figure 4I-L). Interestingly, it appeared that OC1-immunoreactivity was sometimes increased in response to mOtx2 introduction, and may be partially responsible for cooperation with Otx2 in activating the reporter construct (observed in 2 out of 6 spinal cords) (Figure 4K). As was mentioned, OC2 and OC3, which were not visualized in this experiment, also are candidates for cooperation with Otx2. When mOtx2 and mOC1 were both introduced, a clear and robust upregulation of the reporter was observed (Figure 4M-P).

In order to test whether endogenous Thrb might be regulated by these factors, CAG-EGFP and Cag-mOC1 were coelectroporated with or without a CAG-mOtx2 construct. When fluorescent *in situ* hybridization was performed to detect endogenous cThrb mRNA, a clear upregulation of cThrb mRNA was observed only when mOtx2 was present (Figure 4Q-T). This upregulation was not seen in conditions where only mOC1 was electroporated, again suggesting that mOC1 alone is not sufficient to induce cThrb without mOtx2.

Onecut1 and Otx2 occupy the ThrbCRM1 element in vivo

Having shown that Onecut1 and Otx2 regulate the activity of ThrbCRM1 in a co-dependent manner, co-localize in RPCs, and were predicted to bind to the ThrbCRM1 element, we wanted to confirm that these factors did in fact bind *in vivo* to the ThrbCRM1 sequence. In order to test this we used a Chromatin immunoprecipitation (ChIP) assay. The chicken retina was used because of its large size and also because of the large number of Otx2+/Onecut1+ RPCs in this species, presumably correlating with the large number of cone photoreceptors produced during development (data not shown). When antibodies to Otx2 and Onecut1 were used to immunoprecipitate their targets after cross-linking to DNA, a significant enrichment in the ThrbCRM1 element was found for both Otx2 and Onecut1 over that found when a control IgG antibody was used (Figure 4U). Furthermore, a control region lacking predicted Otx2 and Onecut1 binding sites was not significantly enriched in either Otx2 or Onecut1 immunoprecipitates (Figure 4U).

Onecut1 is sufficient to extend the competence window for genesis of cone photoreceptors and HCs to the postnatal period

It could be that mOC1/mOtx2 controls just the expression of Thrb, but it is also possible that these genes act more broadly to establish the RPC[CH] state. To

investigate this possibility, mOC1 was introduced into postnatal mouse RPCs, and the expression of cone and HC markers was assessed. A significant increase in cells positive for Lim1, a specific marker of HCs, was observed in the electroporated population, only when mOC1 was introduced (Figure 5A-J, A'). These cells localized near the vitreal side of the INL layer, where HCs normally go when they are born. Introduction of CAG-mOC1 also resulted in the significant production of RXRγ-positive cells in the developing photoreceptor layer (Figure 5K-T, A'). While RXRγ also marks RGCs, the position and morphology of these cells and their continued expression of Otx2 (data not shown) strongly suggested that these are induced cones. These GFP-positive/ RXRγ -positive cells were also present at P4 and P21 (data not shown). Furthermore, examination of Nr2e3, an exclusive marker of rods in the adult retina (Chen et al., 2005), revealed that mOC1 expression significantly reduced the number of Nr2e3-positive cells in the ONL (Figure 5U-Z, A'). Thus, these data support the notion that expression of mOC1/mOtx2 can shift the competence window such that cones and HCs can be produced in the postnatal period and also suppress the expression of rod photoreceptor markers.

Interference with Onecut transcription activity leads to an increase in rods

The necessity of Onecut factors in retinal development was investigated using methods to reduce their expression and/or activity. Several outcomes were considered. One outcome concerns photoreceptors: would they be made, and would

they be cones? If Onecut family members function like Otx2, then reduction of Onecut activity would lead to fates other than photoreceptors, such as amacrine cells. Alternatively, if absence of Onecut family members allowed for Otx2 to still drive the production of photoreceptors, then rods, but not cones, might be formed. This is the situation in the postnatal mouse retina, when Otx2 is present, but Onecuts are absent from RPCs.

Due to the fact that multiple Onecut family members are coexpressed in RPCs and the high degree of conservation in their DNA-binding domains, it seemed likely that knockdown of each of these genes by RNAi would be necessary to see a phenotype. In order to bypass this requirement, a dominant negative approach was taken. The Engrailed Repressor (EnR) domain was fused to the DNA binding domain of mOC1, either N-terminally (EnR-OC1) or C-terminally (OC1-EnR), and these constructs were introduced into E5 chick retinas. To test whether these fusions were functional, expression from the ThrbCRM1-PLAP reporter was assessed. Both OC1-EnR and EnR-OC1 fusions led to a large decrease in reporter activity compared to the expression of ThrbCRM1 alone (Figure 6A-C, A'-C'). No observable effects on ThrbCRM1 reporter activity were observed when either the EnR domain by itself or the OC1-DBD domain by itself were tested (Figure 6D,E,D',E').

The effects of these dominant negative constructs on cell fates were then assessed. The expression of a coelectroporated Rbp3 element, which is active in both early cone and rods, was tested. In contrast to what was observed with the

ThrbCRM1 reporter, the expression of the Rbp3 reporter was unaffected by OC1-EnR, suggesting that photoreceptors are still generated (data not shown). Interestingly, the Rhodopsin promoter was activated in a premature manner by coelectroporation with OC1-EnR (data not shown). This strongly suggests that interference with Onecut target proteins leads to the premature specification of rods.

In order to test whether endogenous gene expression might support such a model, the expression of L-Maf (also known as MafA) was determined. L-Maf is the earliest marker of rods known in the chicken that allows for the distinction between cone and rods (Ochi et al., 2004). In the mouse and human retina, Nrl is another Maf family transcription factor that in these species is also the earliest known marker of rods (Akimoto et al., 2006). Importantly, these factors are thought to be critical for the expression of rhodopsin, and thus L-Maf up-regulation might explain the effects observed on the Rhodopsin promoter (Rehemtulla et al., 1996; Mears et al., 2001). To determine whether L-Maf could be upregulated in response to OC1-EnR, retinas were electroporated with the EnR constructs and an RNA *in situ* hybridization was performed to detect L-Maf expression. Strikingly, upregulation of L-Maf RNA was observed with two separate probes specific to L-Maf in response to both OC1-EnR and EnR-OC1, compared to either the addition of no EnR construct, or the EnR by itself (Figure 6F-I, F'-I', data not shown). Interestingly, electroporation of just the OC1DBD domain induced the production of some L-Maf positive cells (Figure 6J, J'). This is not unexpected, as the OC1DBD domain could act as a dominant-negative that blocks the binding of endogenous Onecut family members. The upregulation of

L-Maf was weak, and so may explain why an effect on the ThrbCRM1 reporter was not detectable with this construct (Figure 6E). It was also notable that L-Maf positive cells were in a position, and with a morphology, suggestive of early rods. This suggests that OC1-EnR does not upregulate L-Maf in any cell, or convert all RPCs to rods, but may be converting to rods only the cells already delimited by Otx2 and/or other factors to become photoreceptors. Taken together, these results suggest that the OC1-EnR constructs specifically lead to upregulation of L-Maf and likely the rod fate. Thus, Onecut family members may normally function to promote cone genesis and suppress rod genesis in some RPCs and/or in newborn photoreceptors of the embryonic chicken retina.

Alterations in early cone and rod photoreceptor markers in the Onecut1 knockout mouse

To assess whether the apparent photoreceptor fate changes observed in the chicken retina in response to interfering with OC function could be confirmed in the mouse retina, the OC1 KO mouse was examined. Despite the reportedly large overlap in the expression of OC1 and 2 reported in embryonic RPCs, we tested whether these genes might not be completely functionally redundant (Wu et al., 2012). In order to determine if mThrb expression was altered in the OC1 KO mice, both E13.5 and E14.5 retinas from these mice and wild-type control mice were assessed for mThrb RNA expression by RNA *in situ* hybridization. At both E13.5 and

E14.5, there was an observable reduction of Thrb mRNA in the OC1 KO retina (Figure 6K-N). In order to quantify this effect, a qPCR analysis was performed. At both E14.5 and E17.5 a clear reduction in Thrb levels was observed that was statistically significant at E17.5 ($p<0.05$) (Figure 6O). As the OC1-EnR data in the chicken suggested that rod genesis might be upregulated in the OC1 KO mice, the distribution of the early rod marker Nrl was assessed. While it was not possible to detect Nrl mRNA by *in situ* hybridization in either the wild-type or OC1 KO retinas, qPCR experiments revealed that the KOs had a greater than 2-fold increase in the expression of Nrl relative to wildtype mice (Figure 6O). This data was supported by the fact that two independent microarrays of OC1 KO mice also had a greater than 2-fold induction of Nrl compared to wildtype mice at E14.5 (Goetz and Trimarchi, 2014). Thus, as was observed in the chicken retina, the data presented here suggest a critical role for OC factors in controlling the early decision to become a rod or a cone as revealed by alterations in the best characterized markers of these two cell types.

5. Discussion

The current model for photoreceptor development states that Nrl is the key factor that regulates the rod versus cone fate decision (Swaroop et al., 2010). Indeed, there is strong support for the role of Nrl in promoting rod-specific gene expression, whilst suppressing cone-specific programs (Mears et al., 2001; Oh et al., 2007). The model posits that a postmitotic photoreceptor precursor is generated

from RPCs, and the presence or absence of Nrl determines whether that precursor will become a rod or a cone, respectively. A prediction from this model would be that loss of Nrl should lead to upregulation of the earliest markers of cones. However, both this study and previous studies have failed to detect changes in two such markers, RXRγ and/or Thrb, at a time when birthdating studies have shown that approximately 50% of the rods have been generated (Carter-Dawson and LaVail, 1979). The lack of such an effect suggests that there might be a regulatory network upstream of Nrl that is crucial to distinguishing cones and rods at the time of their specification. The data presented here suggest that the Onecut family of factors are indeed such regulatory factors. Both the Onecut1 loss-of-function mutant in the mouse, and Onecut-EnR fusions in the chicken, led to upregulation of Nrl, or the chicken homolog L-Maf, at early developmental times. Onecut factors thus function genetically upstream to suppress Nrl/L-Maf expression and prevent rod photoreceptor development. Interestingly, the ability of the EnR domain to lead to derepression of L-Maf suggests that this may not be a direct effect of Onecut factors on the L-Maf locus, but may be via an as yet unidentified intermediate repressor protein(s). In accord with placing Nrl/L-Maf downstream of Onecut factors, there is no evidence from microarray studies that Onecut factors are upregulated in the Nrl mutant (Yoshida et al., 2004).

While the activity of ThrbCRM1 and its two core regulators, Otx2 and Onecut factors, are co-expressed in the RPC[CH]s, Onecut expression is also detected in Visinin-positive cells of the chicken retina, which are presumed to be photoreceptors at this timepoint (Bruhn and Cepko, 1996; Fischer et al., 2008).

Furthermore, molecular profiling of photoreceptors, characterized by expression of GFP under the control of Crx, has identified Onecut1 as a cone-enriched gene (Muranishi et al., 2010). This suggests that Onecut1 continues to be expressed for some amount of time in newborn cones. The introduction of Onecut1 into the postnatal retina was able to induce early markers of both HCs (Lim1) and cones (RXRγ), suggesting that the RPC[CH] state had been at least partially induced in the postnatal retina. Fully mature cones were not induced, however, and our preliminary evidence suggests that sustained Onecut1 expression from the CAG promoter interferes with progression of photoreceptors beyond the Thrb/RXRγ stage (data not shown). This is not surprising as Onecut1 expression is clearly downregulated in cones by the time they reach the postnatal period (Wu et al., 2012).

In addition to the need for temporal control of Onecut in cones, there may exist other transcriptional networks and signaling mechanisms that are required for the full differentiation of cones. Recent work has identified the Spalt transcription factor member, Sall3, as a regulator of both HC and cone cell differentiation, again revealing a link between these two cell types (de Melo et al., 2011). Sall3 was found to be a potent regulator of more mature markers of these cell types but did not regulate Lim1 or RXRγ, two of the earliest markers that Onecut does regulate (de Melo et al., 2011). Therefore, there may be a transition within the transcription factor network from Onecut factors to Sall3 as cones mature.

Although Onecut1 has been most closely associated with liver gene regulation, several recent studies have identified Onecut Factors as specific cell fate regulators in the spinal cord (motoneurons) and mesencephalic trigeminal nucleus (Samadani and Costa, 1996; Francius and Clotman, 2010; Espana and Clotman, 2012). In the retina, the data presented here suggest that the specificity of Onecut transcriptional targets in the retina is due to cooperation with Otx2. That is, cones and HCs are not generated in the spinal cord, where Onecut factors are found, due to the lack of Otx2. Introduction of Otx2 into the spinal cord is capable of activating the ThrbCRM1 reporter and endogenous Thrb expression. Thus, co-expression of Otx2 and Onecut factors may be sufficient to drive early events in cone genesis, suggesting their applicability in cone induction from stem cells.

The network elements described here may also be conserved in Drosophila. The Otx homologue of Otx, *otd*, is important in Drosophila photoreceptor development (Vandendries et al., 1996). The Drosophila Onecut has DNA-binding domains that are highly related to the vertebrate homologs, and it is also expressed in Drosophila photoreceptors (Nguyen et al., 2000). Moreover, it can bind to Drosophila rhodopsin enhancer sequences (Nguyen et al., 2000). Thus, it will be interesting to explore whether *otd* and Onecut have an evolutionarily conserved role in driving photoreceptor development.

The vertebrate retina comprises approximately 60 different cell types (reviewed in Masland, 2011). This diverse group of cells is generated by a pool of RPCs, many of which were shown to be multipotent throughout development

(Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). While clonal lineage analyses showed the multipotential nature of RPCs, the same analyses showed a great diversity in clone types, originating event from a single time in development. This observation raised the possibility that even though RPCs are multipotent, they may not be equivalent, but may differ in their potential for proliferation, and in their ability to make different cell types. Evidence for distinct types of RPCs has accumulated over the last few years (Godinho et al., 2007; Rompani and Cepko, 2008; Brzezinski et al., 2011; Hafler et al., 2012). In one study, we recently found that embryonic mouse RPCs that express the bHLH transcription factor, Olig2, divide only once, and produce cones and HCs. Some of these 2 cell clones were 2 cones, some were 2 HCs, and some were one HC and one cone. This study has now identified key factors that endow these Olig2+ RPCs with the ability to make these types of daughter cells. Otx2 and Onecut factors are co-expressed in Olig2+ RPCs during early embryonic retinal development, when cones and HCs are made, but are not co-expressed in late embryonic or postnatal Olig2+ RPCs, when rods and amacrine cells are made by Olig2+ RPCs. After cones and HCs are generated, the co-expression of Otx2 and Onecut factors resolves such that cones express Otx2, but not Onecuts, and HCs express Onecuts, but not Otx2. This lack of Otx2 expression in HCs led to an ambiguity in our understanding of the phenotype of the Otx2 KO mouse. The development of HCs, as well as cones, is perturbed in the Otx2 LOF mouse (Nishida et al., 2003; Sato et al., 2007). The reason for this defect in HC development can now be ascribed to a role of Otx2 in the RPC[CH]. There must be a regulatory relationship that resolves the up and down regulation of Onecuts

and Otx2 as differentiation proceeds in cones and HCs. Examination of the CRMs of these and other genes involved in the development of these cell types at these time points should provide candidates for this network.

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7. References

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J.A., Khanna, R., Filippova, E., Oh, E.C.T., Jing, Y., Linares, J.-L., Brooks, M., et al. (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc. Natl. Acad. Sci. U.S.A. *103*, 3890–3895.

Bruhn, S.L., and Cepko, C.L. (1996). Development of the pattern of photoreceptors in the chick retina. J. Neurosci *16*, 1430–1439.

Brzezinski, J.A., 4th, Kim, E.J., Johnson, J.E., and Reh, T.A. (2011). Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development *138*, 3519–3531.

Carter-Dawson, L.D., and LaVail, M.M. (1979). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J. Comp. Neurol *188*, 263–272.

Chen, J., Rattner, A., and Nathans, J. (2005). The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J. Neurosci. *25*, 118–129.

Cherry, T.J., Wang, S., Bormuth, I., Schwab, M., Olson, J., and Cepko, C.L. (2011). NeuroD factors regulate cell fate and neurite stratification in the developing retina. J. Neurosci. *31*, 7365–7379.

Daniele, L.L., Lillo, C., Lyubarsky, A.L., Nikonov, S.S., Philp, N., Mears, A.J., Swaroop, A., Williams, D.S., and Pugh, E.N., Jr (2005). Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the Nrl knockout mouse. Invest. Ophthalmol. Vis. Sci. *46*, 2156–2167.

Edqvist, P.-H.D., and Hallböök, F. (2004). Newborn horizontal cells migrate bi-directionally across the neuroepithelium during retinal development. Development *131*, 1343–1351.

Emerson, M.M., and Cepko, C.L. (2011). Identification of a retina-specific Otx2 enhancer element active in immature developing photoreceptors. Dev. Biol. *360*, 241–255.

Espana, A., and Clotman, F. (2012). Onecut factors control development of the Locus Coeruleus and of the mesencephalic trigeminal nucleus. Mol. Cell. Neurosci. *50*, 93–102.

Fischer, A.J., Foster, S., Scott, M.A., and Sherwood, P. (2008). Transient expression of LIM-domain transcription factors is coincident with delayed maturation of photoreceptors in the chicken retina. J. Comp. Neurol *506*, 584–603.

Fischer, A.J., Wang, S.-Z., and Reh, T.A. (2004). NeuroD induces the expression of visinin and calretinin by proliferating cells derived from toxin-damaged chicken retina. Dev. Dyn. *229*, 555–563.

Francius, C., and Clotman, F. (2010). Dynamic expression of the Onecut transcription factors HNF-6, OC-2 and OC-3 during spinal motor neuron development. Neuroscience *165*, 116–129.

Gehrs, K.M., Anderson, D.H., Johnson, L.V., and Hageman, G.S. (2006). Age-related macular degeneration--emerging pathogenetic and therapeutic concepts. Ann. Med. *38*, 450–471.

Godinho, L., Williams, P.R., Claassen, Y., Provost, E., Leach, S.D., Kamermans, M., and Wong, R.O.L. (2007). Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. Neuron *56*, 597–603.

Hafler, B.P., Surzenko, N., Beier, K.T., Punzo, C., Trimarchi, J.M., Kong, J.H., and Cepko, C.L. (2012). Transcription factor Olig2 defines subpopulations of retinal progenitor cells biased toward specific cell fates. Proc. Natl. Acad. Sci. U.S.A. *109*, 7882–7887.

Hartong, D.T., Berson, E.L., and Dryja, T.P. (2006). Retinitis pigmentosa. Lancet *368*, 1795–1809.

Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. Development *128*, 1313–1322.

Holt, C.E., Bertsch, T.W., Ellis, H.M., and Harris, W.A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron *1*, 15–26.

Hsiau, T.H.-C., Diaconu, C., Myers, C.A., Lee, J., Cepko, C.L., and Corbo, J.C. (2007). The cis-regulatory logic of the mammalian photoreceptor transcriptional network. PLoS ONE *2*, e643.

Jadhav, A.P., Mason, H.A., and Cepko, C.L. (2006). Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development *133*, 913–923.

Jones, I., Ng, L., Liu, H., and Forrest, D. (2007). An intron control region differentially regulates expression of thyroid hormone receptor beta2 in the cochlea, pituitary, and cone photoreceptors. Mol. Endocrinol. *21*, 1108–1119.

Koike, C., Nishida, A., Ueno, S., Saito, H., Sanuki, R., Sato, S., Furukawa, A., Aizawa, S., Matsuo, I., Suzuki, N., et al. (2007). Functional roles of Otx2 transcription factor in postnatal mouse retinal development. Mol. Cell. Biol *27*, 8318–8329.

Liu, W., Wang, J.H., and Xiang, M. (2000). Specific expression of the LIM/homeodomain protein Lim-1 in horizontal cells during retinogenesis. Dev. Dyn *217*, 320–325.

Masland, R.H. (2011). Cell Populations of the Retina: The Proctor Lecture. IOVS *52*, 4581–4591.

Matsuda, T., and Cepko, C.L. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc. Natl. Acad. Sci. U.S.A *101*, 16–22.

Matys, V., Kel-Margoulis, O.V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., et al. (2006). TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res. *34*, D108–110.

Mears, A.J., Kondo, M., Swain, P.K., Takada, Y., Bush, R.A., Saunders, T.L., Sieving, P.A., and Swaroop, A. (2001). Nrl is required for rod photoreceptor development. Nat. Genet. *29*, 447–452.

de Melo, J., Peng, G.-H., Chen, S., and Blackshaw, S. (2011). The Spalt family transcription factor Sall3 regulates the development of cone photoreceptors and retinal horizontal interneurons. Development *138*, 2325–2336.

Mori, M., Ghyselinck, N.B., Chambon, P., and Mark, M. (2001). Systematic immunolocalization of retinoid receptors in developing and adult mouse eyes. Invest. Ophthalmol. Vis. Sci. *42*, 1312–1318.

Muranishi, Y., Sato, S., Inoue, T., Ueno, S., Koyasu, T., Kondo, M., and Furukawa, T. (2010). Gene expression analysis of embryonic photoreceptor precursor cells using BAC-Crx-EGFP transgenic mouse. Biochem. Biophys. Res. Commun. *392*, 317–322.

Muranishi, Y., Terada, K., Inoue, T., Katoh, K., Tsujii, T., Sanuki, R., Kurokawa, D., Aizawa, S., Tamaki, Y., and Furukawa, T. (2011). An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. J. Neurosci. *31*, 16792–16807.

Ng, L., Hurley, J.B., Dierks, B., Srinivas, M., Saltó, C., Vennström, B., Reh, T.A., and Forrest, D. (2001). A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat. Genet. *27*, 94–98.

Ng, L., Ma, M., Curran, T., and Forrest, D. (2009). Developmental expression of thyroid hormone receptor beta2 protein in cone photoreceptors in the mouse. Neuroreport *20*, 627–631.

Nguyen, D.N., Rohrbaugh, M., and Lai, Z. (2000). The Drosophila homolog of Onecut homeodomain proteins is a neural-specific transcriptional activator with a potential role in regulating neural differentiation. Mech. Dev. *97*, 57–72.

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., and Furukawa, T. (2003). Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat. Neurosci *6*, 1255–1263.

Ochi, H., Sakagami, K., Ishii, A., Morita, N., Nishiuchi, M., Ogino, H., and Yasuda, K. (2004). Temporal expression of L-Maf and RaxL in developing chicken retina are arranged into mosaic pattern. Gene Expr. Patterns *4*, 489–494.

Oh, E.C.T., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007). Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc. Natl. Acad. Sci. U.S.A. *104*, 1679–1684.

Ong, J.M., and da Cruz, L. (2012). A review and update on the current status of stem cell therapy and the retina. Br. Med. Bull. *102*, 133–146.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D.J., and Swaroop, A. (1996). The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc. Natl. Acad. Sci. U.S.A. *93*, 191–195.

Roberts, M.R., Hendrickson, A., McGuire, C.R., and Reh, T.A. (2005). Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. Invest. Ophthalmol. Vis. Sci. *46*, 2897–2904.

Roe, T., Reynolds, T.C., Yu, G., and Brown, P.O. (1993). Integration of murine leukemia virus DNA depends on mitosis. EMBO J. *12*, 2099–2108.

Rompani, S.B., and Cepko, C.L. (2008). Retinal progenitor cells can produce restricted subsets of horizontal cells. Proc. Natl. Acad. Sci. U.S.A *105*, 192–197.

Samadani, U., and Costa, R.H. (1996). The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. Mol. Cell. Biol. *16*, 6273–6284.

Sato, S., Inoue, T., Terada, K., Matsuo, I., Aizawa, S., Tano, Y., Fujikado, T., and Furukawa, T. (2007). Dkk3-Cre BAC transgenic mouse line: a tool for highly efficient gene deletion in retinal progenitor cells. Genesis *45*, 502–507.

Swain, P.K., Hicks, D., Mears, A.J., Apel, I.J., Smith, J.E., John, S.K., Hendrickson, A., Milam, A.H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J. Biol. Chem. *276*, 36824–36830.

Swaroop, A., Kim, D., and Forrest, D. (2010). Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. Nat. Rev. Neurosci. *11*, 563–576.

Tian, E., Kimura, C., Takeda, N., Aizawa, S., and Matsuo, I. (2002). Otx2 is required to respond to signals from anterior neural ridge for forebrain specification. Dev. Biol. *242*, 204–223.

Trimarchi, J.M., Harpavat, S., Billings, N.A., and Cepko, C.L. (2008). Thyroid hormone components are expressed in three sequential waves during development of the chick retina. BMC Dev. Biol *8*, 101.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J. Comp. Neurol. *502*, 1047–1065.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Vandendries, E.R., Johnson, D., and Reinke, R. (1996). orthodenticle is required for photoreceptor cell development in the Drosophila eye. Dev. Biol *173*, 243–255.

West, E.L., Gonzalez-Cordero, A., Hippert, C., Osakada, F., Martinez-Barbera, J.P., Pearson, R.A., Sowden, J.C., Takahashi, M., and Ali, R.R. (2012). Defining the Integration Capacity of Embryonic Stem Cell-Derived Photoreceptor Precursors. STEM CELLS *30*, 1424–1435.

Wetts, R., and Fraser, S.E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142–1145.

Wu, F., Sapkota, D., Li, R., and Mu, X. (2012). Onecut 1 and Onecut 2 are potential regulators of mouse retinal development. J. Comp. Neurol. *520*, 952–969.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development *133*, 1367–1378.

Yoshida, S., Mears, A.J., Friedman, J.S., Carter, T., He, S., Oh, E., Jing, Y., Farjo, R., Fleury, G., Barlow, C., et al. (2004). Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum. Mol. Genet. *13*, 1487–1503.

Young, R.W. (1985). Cell differentiation in the retina of the mouse. Anat. Rec. *212*, 199–205.

8. Figures and Legends

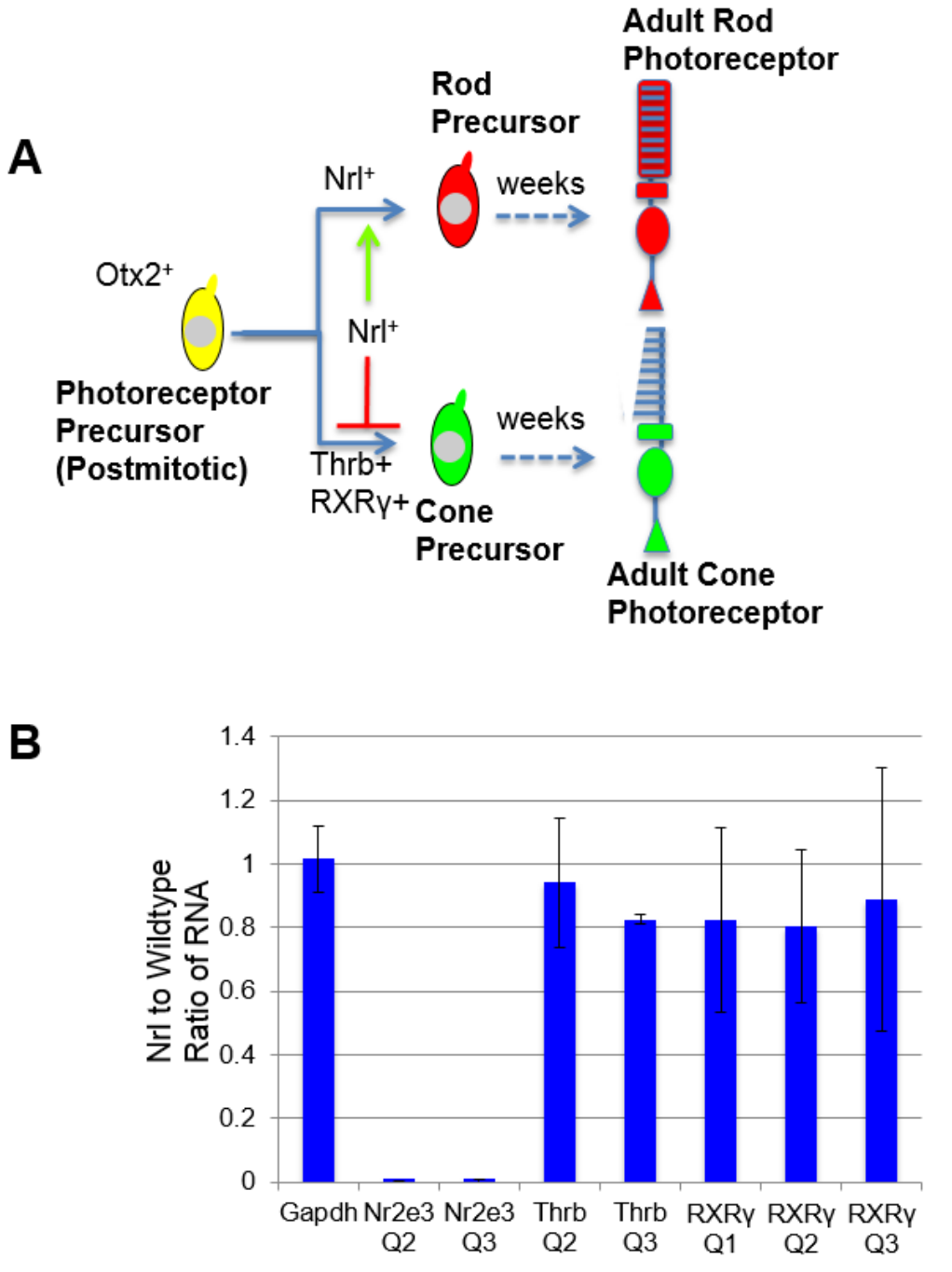

Figure 1. Examination of Photoreceptor mRNA Levels in Nrl Knockout Retinas

(A) A current model of photoreceptor specification (based on Swaroop et al., 2010). Otx2 has been shown to be required for the genesis of both rods and cones. Nrl has been proposed to determine whether a cell is a rod or a cone, acting in a postmitotic precursor photoreceptor cell. In the presence of Nrl, precursors are directed to become rods while in the absence of Nrl, these cells become cones. Both of these cell types acquire their final phenotype over the course of several weeks. (B) qPCR analysis of retinal cDNA at P0. Plot shows the fold difference of relative RNA levels between wild-type and Nrl mice and the error bars represent the standard deviation (see methods).

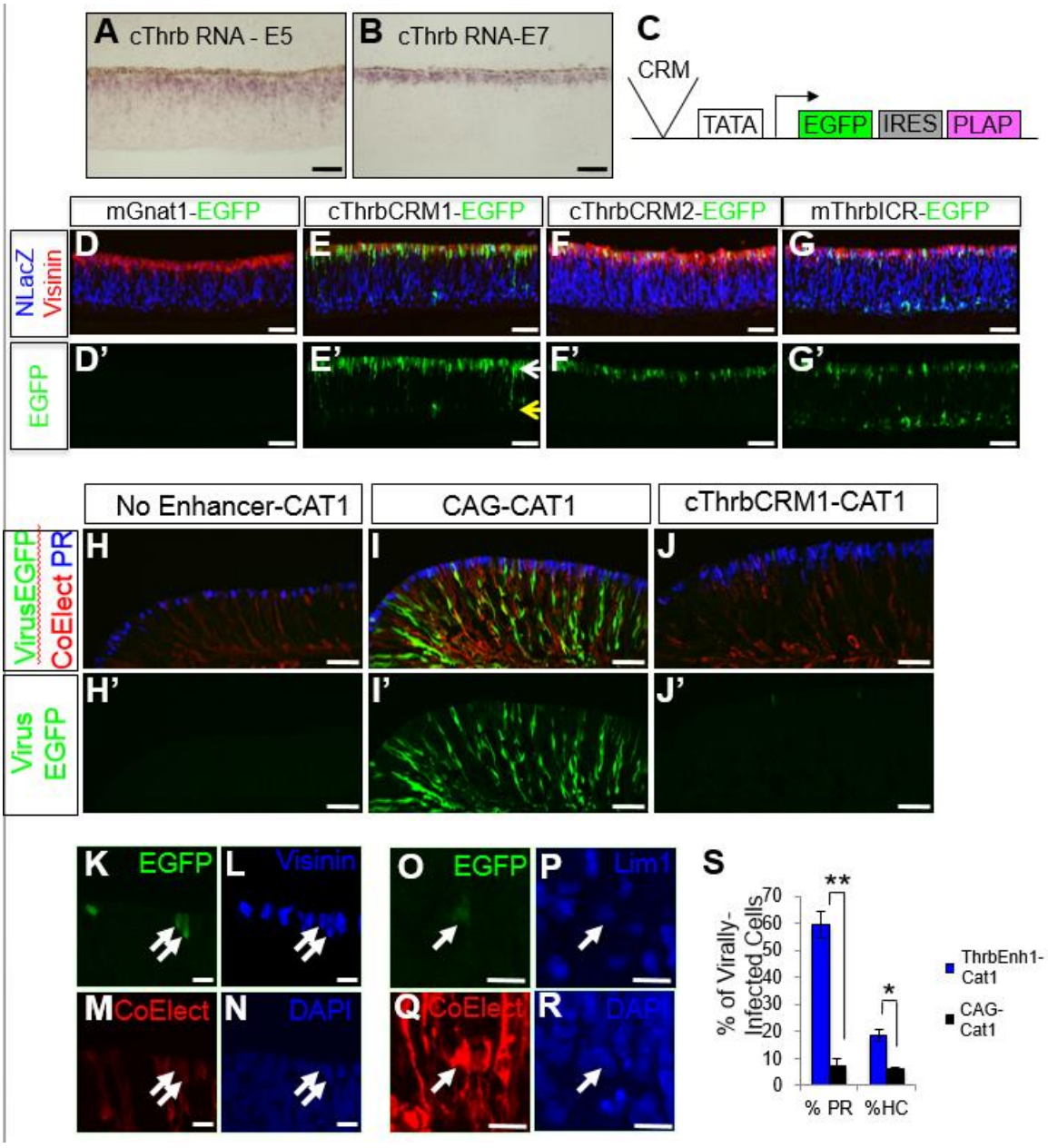

Figure 2. Identification and Analysis of Thrb CRMs

(A, B) RNA *in situ* hybridization for cThrb at E5 (A) and E7 (B) in central chicken retina. (C) Schematic of the Stagia3 reporter vector. (D-G) E5 retinas co-electroporated with CAG-nβgal plasmids and Stagia3 plasmids, cultured for two days, sectioned, and immunofluorescently stained with αEGFP (green), αVisinin (red), and αβgal (blue). Elements cloned into Stagia 3 were mGnat1 5' sequences (D), cThrbCRM1 (E), cThrbCRM2 (F), and mThrbICR (G), . (D'-G') EGFP channels alone of above images. White arrow in E' shows the position of GFP+ cells just vitread to the scleral surface and the yellow arrow the GFP+ cells just sclerad to the vitreal surface. (H-J) Sections of E5 retinas co-electroporated with CAG-AU1Gapdh

(to mark electroporated cells – AU1 is an epitope tag, Emerson and Cepko, 2011) and CAT1 receptor constructs, infected with GFP expressing retrovirus 6 hours later and harvested 40 hours later. Antigens detected were EGFP (green), AU1 (red), and Visinin (blue). CAT1 plasmids used were either a no enhancer negative control (H), CAG positive control (I) or ThrbCRM1 (J). EGFP channels alone of above images are in H'-J'. (K-N) Magnified views of ThrbCRM1-CAT1 retinas detected for viral EGFP (K), Visinin (L), AU1 (M), and DAPI (N). (O-R) Magnified views of ThrbCRM1-CAT1 retinas detected for EGFP (O), Lim1 (P), AU1 (Q), and DAPI (R). (S) Percentage of virally transduced EGFP positive cells that were identified as photoreceptors (Visinin+) or HCs (Lim1+) when the CAT1 receptor was under the control of the ThrbCRM1 element (green bars) or the CAG element (red bars). Values represent the average of the data from three biological replicates and a two tailed student's t-test was used to assess statistical significance (** represents $p<0.001$ and * represents $p<0.005$). Scale bar: 40μm for A,B,D-G,D'-G', H-J, H'-J'; 10μm for K-R.

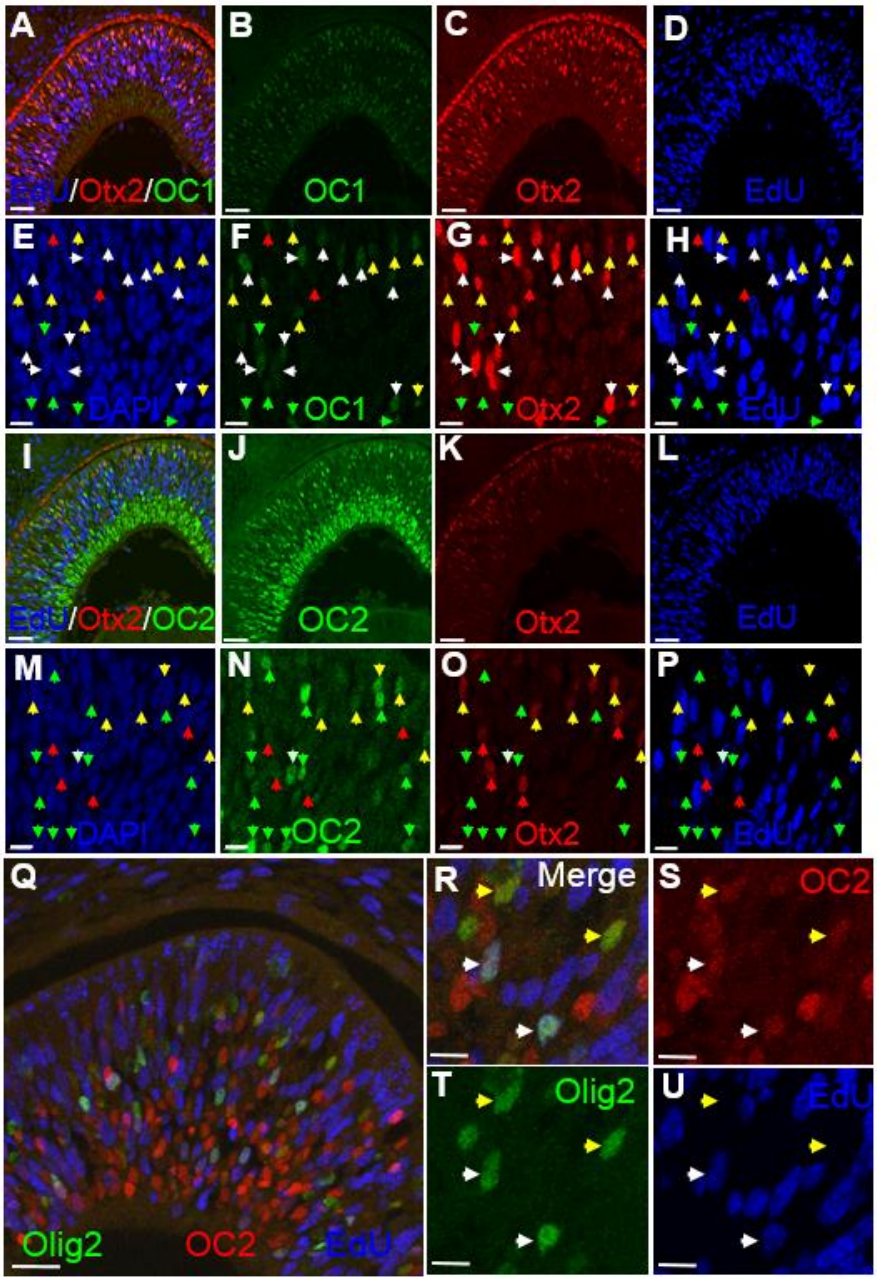

Figure 3. Otx2 is Co-expressed with Onecut1 and Onecut2 in Mouse RPCs and Onecut2 is Expressed in Embryonic Olig2+ RPCs.

Sections of mouse retinas from E13.5 embryos exposed *in utero* to EdU for 2.5 hours before harvest, processing, and confocal imaging. (A-D) Imaged for OC1 (green), Otx2 (red), EdU (blue) and DAPI (not shown). (A) Merge of the three channels (B) OC1 C) Otx2 D) EdU (E-H) Single z-plane images of each channel as denoted. Green arrows ($OC1^+$ only), red arrows ($Otx2^+$ only), yellow arrows ($Otx2^+/OC1^+$), and white arrows ($Otx2^+/OC1^+/EdU^+$) point to specific cells. White * marks Otx2+ RPE

and yellow * marks OC1+ retinal ganglion cell layer. (I-L) Imaged for OC2 (green), Otx2 (red), EdU (blue) and DAPI (not shown). (I) Merge of the three channels. (J) Otx2 (K) OC2 (L) EdU (M-P) Single z-plane images of each channel as denoted. Arrows and asterisks same as in E-H, but with OC2 instead of OC1. (Q) Imaged for Olig2 (green), OC2 (red) and EdU (blue). (P-R) High magnification view of retina processed as in panel O with channels denoted on the panel. White arrows ($Olig2^+/OC2^+/EdU^+$) and yellow arrows ($Olig2^+/OC2^+$) point to specific cells. Scale bar: 40μm for A-D,I-L,Q; 10μm for E-H, M-P, R-U.

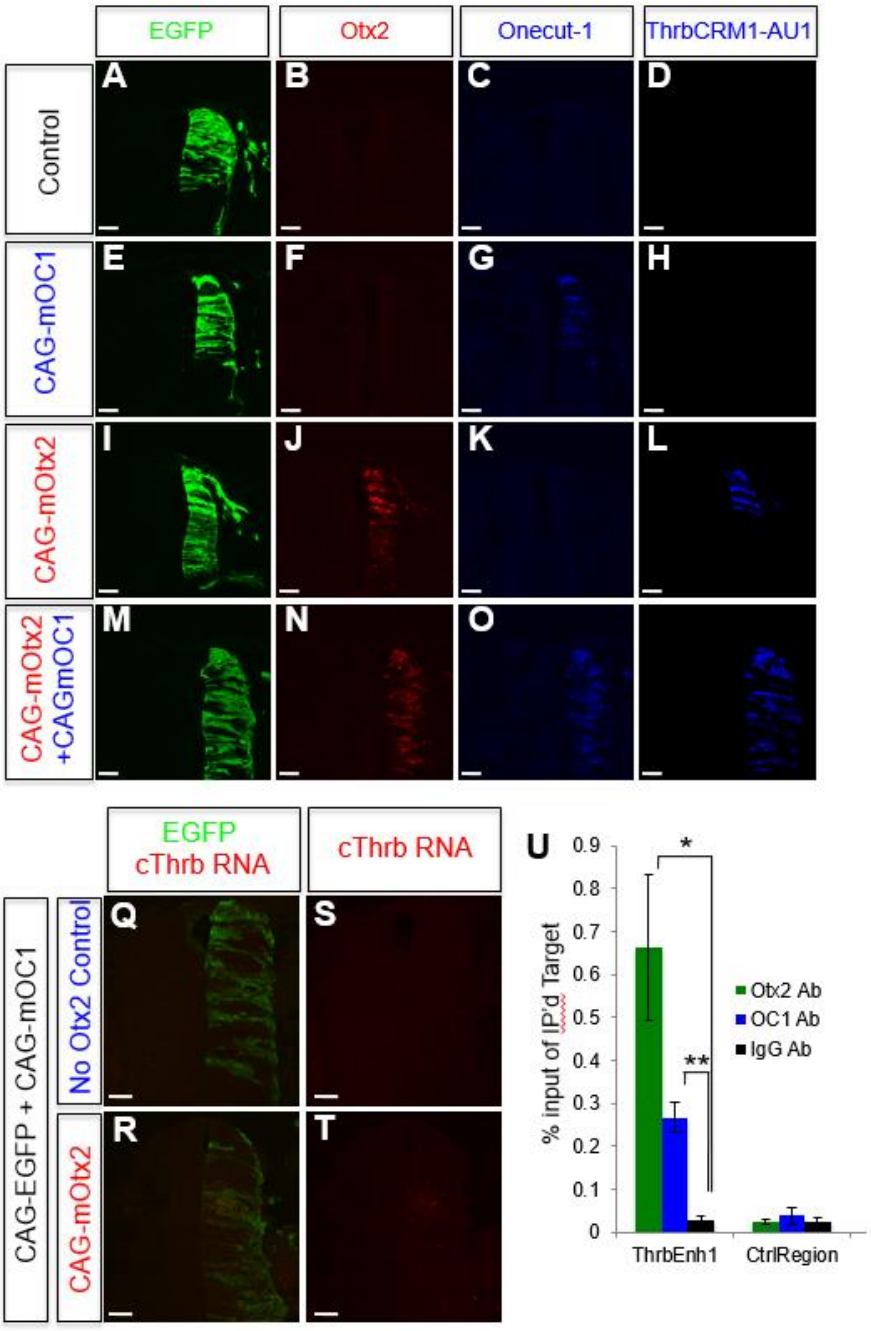

Figure 4. Effects of Spinal Cord Misexpression of mOC1 and mOtx2 on the ThrbCRM1 Reporter and cThrb Endogenous Gene Expression.

(A-P) Chicken spinal cords electroporated at E3 with CAG-EGFP, ThrbCRM1-AU1 and the misexpression plasmid listed to the far left of each row. Sections of spinal cord were immunofluorescently labeled for detection of EGFP (green), Otx2 (red), Onecut-1 (blue) and AU1 (blue) and are noted at the top of each column. (Q,R) Spinal cords electroporated with the plasmids shown to the left of the rows and processed for detection of cThrb RNA (red) and EGFP (green). (S,T) cThrb RNA

signal by itself. Dorsal is located at the top of the section. Similar results were found for N≥3 spinal cords for each experiment. (U) Plot of the amount of the target DNA region noted on the X-axis immunoprecipitated by antibodies to Otx2 (green bars), OC1 (blue bars), and normal rabbit IgG (black bars). The Y-axis represents amount precipitated as percentage of total chromatin input. Bars represent the averages of three biological triplicates for each condition and error bars the standard error of the mean (S.E.M.). A two tailed student's t-test revealed statistically significant differences between some samples (* represents $p< 0.02$; ** represents $p<0.005$). Scale bar: 40μm

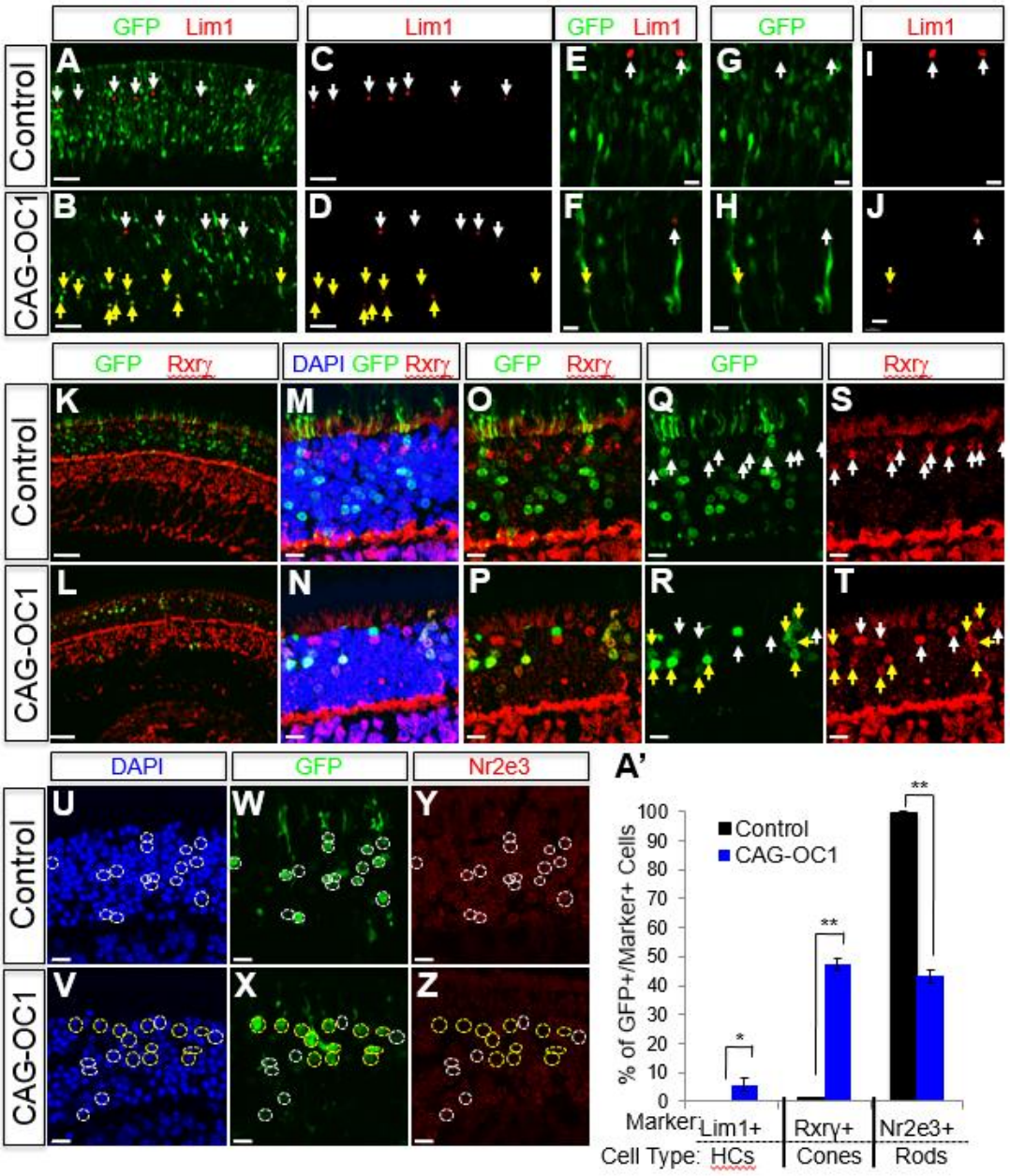

Figure 5. mOC1 Misexpression in the Postnatal Mouse Retina Induces HC and Cone Gene Expression and Inhibits Rod Differentiation.

Mouse P0 retinas *in vivo* electroporated with CAG-EGFP with or without CAG-mOC1 and then harvested, sectioned, and processed for confocal imaging. The channels shown in each panel are denoted at the top of each column and the presence or absence of mOC1 is denoted to the left of each row. (A-J) Retinas harvested at P4 and processed for EGFP (green), Lim1 (red), and DAPI (not shown). White arrows point to Lim1^{+} cells that were not electroporated (EGFP^{-}), corresponding to the

normally generated HCs, yellow arrows point to EGFP$^+$/Lim1$^+$ cells. (E-J) Single z-section images. (K-T) Retinas harvested at P30 and processed for EGFP (green), RXRγ (red), and DAPI (blue). White arrows point to RXRγ$^+$ cells that were not electroporated (EGFP$^-$) corresponding to normally generated cones and yellow arrows point to EGFP$^+$/RXRγ$^+$ cells. (U-Z) Retinas harvested at P30 and processed for EGFP (green), Nr2e3 (red), and DAPI (blue). White dotted lines encompass EGFP$^+$ nuclei that are Nr2e3$^+$. Yellow dotted lines encompass GFP$^+$ nuclei that are Nr2e3$^-$. (A') Quantitation of the overlap of CAG-GFP with the marker listed along the X-axis in either Control or CAG-OC1 electroporated retinas (as in panels A-Z). The cell type normally associated with that marker expression and location is given below the markers. Values represent the average from three retinas and error bars represent the S.E.M.. A student's t-test was used to assess statistical significance (* represents $p<0.05$ using a one-tailed t-test; ** represents $p<0.0001$ using a two tailed t-test). Similar results were found for $N\geq3$ retinas for each set of markers. GFP signal represents only the intrinsic fluorescence of GFP and was not immuno-amplified. Scale bar: 10μm E-J, M-T, W-D'; 40μm A-D, K, L, U, V.

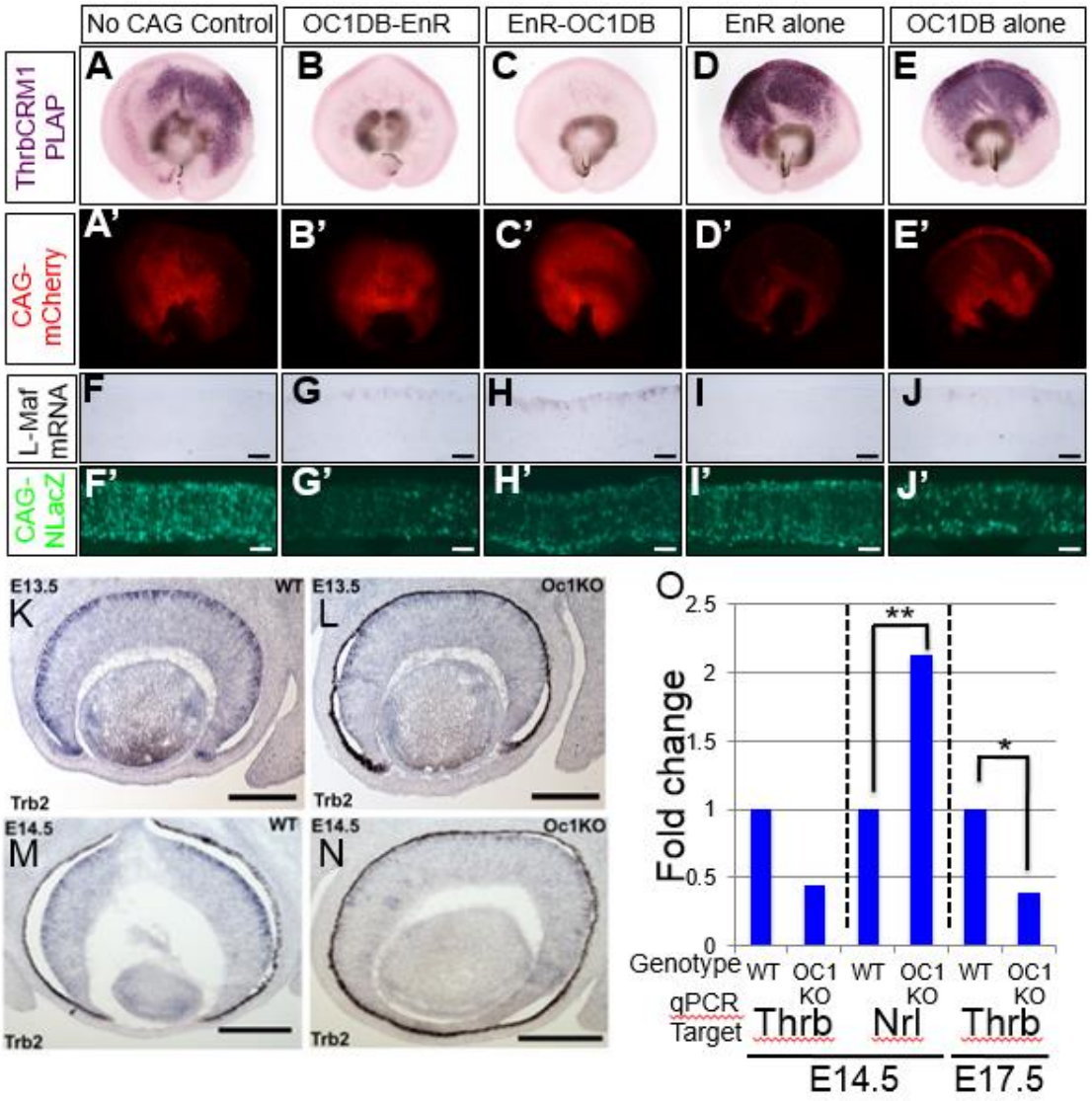

Figure 6. Interference with Onecut Transcriptional Activity in Both Chickens and Mice Leads to Downregulation of ThrbCRM1/Thrb mRNA expression and Upregulation of the Rod Photoreceptor Gene Nrl/L-Maf.

(A-E) E5 chicken retinas electroporated with CAG-mCherry, ThrbCRM1-PLAP, and the CAG construct noted at the top of each column and developed for PLAP activity 2 days later. Similar results were found with biological replicates. (A'-E') CAG-mCherry visualization of the retinas shown above. (F-J) E5 retinas electroporated with CAG-nβgal and the CAG construct at the top of each column, cultured for three days and processed for RNA *in situ* hybridization to detect L-Maf as shown by AP staining. Biological replicates and experiments with a different probe to L-Maf gave the same result. (F'-J') Immunofluorescent detection of βgal for the sections shown

above in F-J. (K-N) RNA *in situ* hybridization to detect mThrb expression in the embryonic retina of wildtype (K,M) and OC1 KO mice (L,N) at E13.5 and E14.5. qPCR analysis of Thrb and Nrl expression in the embryonic retinas of wildtype and OC1 KO mice at E14.5 and E17.5. $N \geq 3$ animals per genotype. * denotes $p<0.05$ and ** denotes $p<0.001$ using a two-tailed t-test. Scale bar: 25μm for F-J,F'-J'; 100μm for K-N.

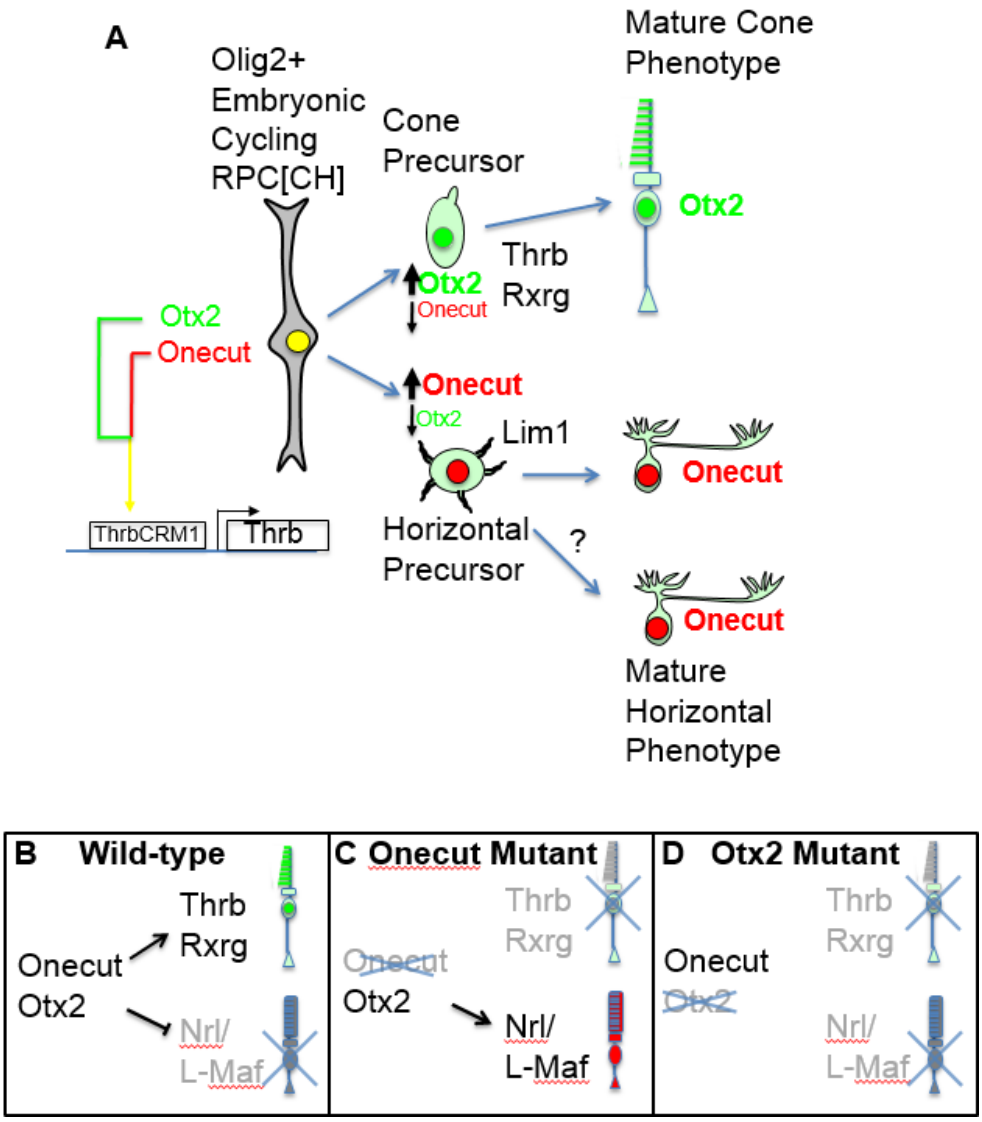

Figure 7. Models for the Roles of Otx2 and Onecut Factors in Horizontal Cell and Photoreceptor Genesis.

(A) A model for Onecut action in the retina. Otx2 is coexpressed with Onecut1 and Onecut2 in RPC[CH]s as defined by their lineage and by their coexpression with Olig2 at E13.5. Together, Otx2 and Onecut factors upregulate Thrb through the ThrbCRM1 element. The RPC[CH] divides to give rise to cones and HCs through an undetermined number of intermediary divisions. In cone precursor cells, the level of Onecut factors declines while in HC precursors, the level of Onecut increases and Otx2 decreases. (B) In Wild-type RPCs, coexpression of Otx2 and Onecut factors promotes cone genesis and inhibits rod genesis. (C) In Onecut Mutants, rod genesis is promoted and cone genesis is impaired. (D) In Otx2 Mutants, all photoreceptor genesis is inhibited and amacrine cells are generated instead.